Genetic diversity of *Ampelomyces* mycoparasites isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences

Chen Liang[1], Jiarong Yang[2], Gábor M. Kovács[3], Orsolya Szentiványi[4], Baodu Li[1], XiangMing Xu[5] and Levente Kiss[4*]

[1]Plant Protection Department, Laiyang Agricultural College, Chunyang Road, Chengyang, Qingdao, 266109, Shandong Province, PR China
[2]Institute of Crop Protection, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shaanxi Province, PR China
[3]Eötvös Loránd University, Department of Plant Anatomy, H-1117 Budapest, Pázmány Péter sétány 1/C, Hungary
[4]Plant Protection Institute of the Hungarian Academy of Sciences, H-1525 Budapest, PO Box 102, Hungary
[5]East Malling Research, East Malling, Kent, ME19 6BJ, UK

Liang, C., Yang, J., Kovács, G.M., Szentiványi, O., Li, B., Xu, X.M. and Kiss, L. (2007). Genetic diversity of *Ampelomyces* mycoparasites isolated from different powdery mildew species in China inferred from analyses of rDNA ITS sequences. Fungal Diversity 24: 225-240.

Pycnidial fungi belonging to the genus *Ampelomyces* are common intracellular mycoparasites of the *Erysiphaceae* worldwide. As a part of a project which aimed to isolate and test potential biocontrol agents of powdery mildew infections of economically important crops in China, a total of 23 *Ampelomyces* isolates were obtained from many different species of the *Erysiphaceae* in five provinces of China. In addition, four new *Ampelomyces* isolates were obtained in Europe for this study. Mycoparasitic tests showed that all the 27 new isolates produced intracellular pycnidia in the conidiophores of *Podosphaera xanthii* and/or *Golovinomyces orontii* when these powdery mildew species were inoculated with conidial suspensions of the isolates. This confirmed that the new isolates can be identified as *Ampelomyces* mycoparasites and they were not confused with other pycnidial mycoparasites of powdery mildew fungi. The ITS sequence of the nuclear rRNA gene of the 27 new isolates were analyzed together with 20 sequences of other *Ampelomyces* isolates determined in earlier studies. The ITS sequences of some isolates obtained in China were identical with those of some European and/or North American isolates which indicates a global distribution of these mycoparasites. At the same time, 16 Chinese isolates formed a distinct group, which was only distantly related to the already known groups of the European and the North American *Ampelomyces* isolates. *Ampelomyces* mycoparasites with similar or identical ITS sequences were found in different powdery mildew hosts in China. Also, mycoparasites with different ITS sequences were isolated from the same powdery mildew species during this study. Thus,

*Corresponding author: L. Kiss; e-mail: LKISS@NKI.HU

no correlation was found between the ITS sequences of the mycoparasites and the host fungi and host plants where they came from.

Key words: biocontrol agents, *Erysiphaceae*, phylogenetic analysis, rDNA ITS sequences

Introduction

Pycnidia of *Ampelomyces* are commonly found inside the hyphae, conidiophores, conidia and immature ascomata of powdery mildew fungi worldwide (Falk *et al*., 1995; Kiss, 1998). Their conidia are released from intracellular pycnidia by the rupture of both the pycnidial and the powdery mildew cell walls, then germinate on host plant surfaces, penetrate the powdery mildew hyphae found in their vicinity, and continue their growth internally, from cell to cell through the septal pores, destroying the invaded parts of the mildew mycelia and producing new intracellular pycnidia 5-7 days after penetration (Hashioka and Nakai, 1980; Sundheim and Krekling, 1982). These mycoparasites can also be transported for long distances by wind within the parasitized powdery mildew conidia (Speer, 1978; Sundheim, 1982). Cross-inoculation experiments have repeatedly demonstrated that *Ampelomyces* mycoparasites collected from a given powdery mildew species can produce intracellular pycnidia in mycelia of other species of the Erysiphaceae (De Bary, 1870; Philipp and Crüger, 1979; Sztejnberg *et al*., 1989). Their biology and biocontrol potential have recently been reviewed by Kiss *et al*. (2004).

Ampelomyces mycoparasites were mostly studied for their use as biological control agents (BCAs) of powdery mildew infections of various crops (Sztejnberg *et al*., 1989; Chen and Yang, 1990; Paulitz and Bélanger, 2001; Bélanger and Labbé, 2002; Kiss, 2003; Kiss *et al*., 2004; Liang *et al*., 2004). A few *Ampelomyces* strains have already been commercialized as the active ingredients of biofungicide products in different parts of the world. In the USA and some European countries, such a product was registered under the trade name ‘AQ10 Biofungicide®’ to be used in the control of grape powdery mildew and a few other economically important powdery mildew diseases (Hofstein *et al*., 1996; Whipps and Lumsden, 2001). More recently, an *Ampelomyces* strain has been developed as ‘Q-fect WP’ in Korea (Lee *et al*., 2004) and another one has been started to be produced in India as ‘Stanes Bio-Dewcon’ (S. Rarmarethinam, pers. comm).

Although current practice is to consider that all pycnidial mycoparasites of powdery mildews belong to one single species, namely *Ampelomyces quisqualis*, and no attention has been paid to the possible differences among various strains used in biocontrol experiments (Kiss, 2003), molecular analyses have shown that these intracellular mycoparasites are genetically diverse (Kiss,

1997; Kiss and Nakasone, 1998; Sullivan and White, 2000; Nischwitz *et al*., 2005; Szentiványi *et al*., 2005). All these studies were based on the analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (nrDNA). Genetically different *Ampelomyces* strains were isolated from the same powdery mildew species (Kiss, 1997) and, on the other hand, strains with identical rDNA ITS sequences were obtained from different mycohost and host plant species (Szentiványi *et al*., 2005). The differences in the rDNA ITS sequences of various *Ampelomyces* mycoparasites suggest that the binomial *'A. quisqualis'* should be regarded as a name of a problematic species complex (Kiss and Nakasone, 1998). In fact, there are more than 40 species of *Ampelomyces* described in the older mycological literature (Sutton, 1980), but these are not currently in use. Molecular analyses carried out by Kiss and Nakasone (1998), Sullivan and White (2000) and Szentiványi *et al.* (2005) did not support the species status of some of the *Ampelomyces* taxa proposed by Rudakov (1979). Thus, until the species are clearly delineated within the genus *Ampelomyces*, the use of '*Ampelomyces* spp.' was recommended (Kiss, 1997). In addition, Sullivan and White (2000) showed that some fungi identified as *'Ampelomyces'* were confused with *Phoma glomerata*, another pycnidial mycoparasite of powdery mildews.

The natural occurrence of *Ampelomyces* in various species of the *Erysiphaceae* has long been reported from different parts of Asia, for example from Japan (Hino and Kato, 1929), China (Tai, 1979; Chen and Yang, 1990; Liang *et al*., 2004), India (Mhaskar and Rao, 1974; Belsare *et al*., 1980), Taiwan (Tsay and Tung, 1991) and Korea (Shin, 1994; Shin and Kyeung, 1994; Lee, 1999). However, none of the Asian isolates of *Ampelomyces* have been included in any molecular analyses to date.

As a part of a project which aimed to obtain and test potential BCAs against powdery mildew infections of economically important crops in China, we have isolated a large number of pycnidial mycoparasites from different powdery mildew species in different parts of the country (Liang, 2004). The objectives of this study were to (i) identify the Chinese isolates based on their morphological and cultural characteristics, and mycoparasitic activity assessed in detached leaf cultures, (ii) determine the rDNA ITS sequences in these isolates, and (iii) investigate their phylogenetic relationships with other pycnidial mycoparasites of powdery mildews based on ITS sequence analyses.

Materials and methods

Isolates

Powdery mildew-infected leaves and stems were collected from as many plant species as possible in different parts of China between 2002-2004 and examined under a stereomicroscope for the presence of intracellular pycnidia characteristic of *Ampelomyces* in the mildew mycelia. When found, one or two pycnidia were removed with sterile hand-made glass needles (Goh, 1999) from the mildew mycelia and were put on potato dextrose agar (PDA) supplemented with 0.5% chloramphenicol. To produce pure cultures of these fungi, the emerging colonies were transferred to new plates as soon as they started to grow on the media. Pure cultures were maintained on PDA at room temperature and transferred every 6-8 weeks to new plates.

The dimensions of pycnidia and conidia were determined before isolation using light microscopy. Radial growth of isolates in culture was determined by measuring every third day the diameter of colonies inoculated as mycelial discs of 10 mm diameter and kept on PDA at 22ºC. The morphological and cultural characteristics of the Chinese isolates obtained in this study were compared with the same patterns of six authentic *Ampelomyces* isolates (CBS 130.79, ATCC 201056, DSM 2222, MYA 3389, AQ10 and 263) obtained from culture collections and other sources. The designations, host plants, host fungi and place and year of isolation of the new isolates are given in Table 1 while the same data for the authentic *Ampelomyces* isolates are included in Table 2. Powdery mildew nomenclature follows the new system proposed by Braun and Takamatsu (2000) and Braun *et al*. (2002). Figure 1 indicates the locations where the Chinese isolates come from.

Mycoparasitic tests

Two sets of experiments were carried out in China and in Hungary, respectively, to examine whether the newly obtained isolates produce intracellular pycnidia in the conidiophores of powdery mildew fungi and, thus, can be identified as *Ampelomyces*. In China, at Laiyang Agricultural College, detached cucumber leaves infected with *Podosphaera xanthii*, and kept with their petioles in water, were inoculated with conidial suspensions of a total of 19 new isolates (HMLAC201-HMLAC222) as described in Szentiványi *et al*. (2005). The inoculated leaves were incubated in transparent plastic boxes in the laboratory for 7-10 days and then examined under a stereomicroscope for the presence of intracellular pycnidia in the powdery mildew mycelia. In

Table 1. Designations, host fungi, host plants, place and year of isolation and ITS sequence database accession numbers of the *Ampelomyces* isolates obtained during this study and included in the phylogenetic analysis.

Isolate designation	Host fungus	Host plant	Place and year of isolation	ITS sequence database accession number
HMLAC201	*Podosphaera fusca*	*Coreopsis grandiflora*	Laiyang, Shandong, 2002	DQ490745
HMLAC202	*Arthrocladiella mougeotii*	*Lycium chinense*	Laiyang, Shandong, 2002	DQ490746
HMLAC203	*P. fusca*	*Arctium lappa*	Laiyang, Shandong, 2002	DQ490747
HMLAC204	*Erysiphe pisi*	*Vigna sesquipedalis*	Laiyang, Shandong, 2002	DQ490758
HMLAC206	*P. fusca*	*Ixeris chinensis*	Laiyang, Shandong, 2002	DQ490760
HMLAC207	*P. xanthii*	*Cucurbita maxima*	Laiyang, Shandong, 2002	DQ490759
HMLAC208	*P. fusca*	*Xanthium sibiricum*	Laiyang, Shandong, 2002	DQ490754
HMLAC209	*P. ferruginea*	*Sanguisorba officinalis*	Laiyang, Shandong, 2002	DQ490763
HMLAC210	*P. xanthii*	*Cucumis sativus*	Laiyang, Shandong, 2002	DQ490755
HMLAC211	*P. fusca*	*Helianthus annuus*	Jinan, Shandong, 2002	DQ490756
HMLAC212	*Phyllactinia fraxini*	*Fraxinus excelsior*	Köln, Germany, 2002	DQ490764
HMLAC214	*E. sordida*	*Plantago major*	Köln, Germany, 2002	DQ490765
HMLAC216	*Oidium* sp.	*Castanopsis* sp.	Guangzhou, Guangdong, 2002	DQ490767
HMLAC217	*P. xanthii*	*Cucurbita moschata*	Yaan, Sichuan, 2002	DQ490752
HMLAC218	*Oidium* sp.	*Euonymus japonicus*	Yaan, Sichuan, 2002	DQ490768
HMLAC219	*P. fusca*	*Sechium edule*	Yaan, Sichuan, 2002	DQ490757
HMLAC220	*P. fusca*	*Sonchus oleraceus*	Weihai, Shandong, 2002	DQ490753
HMLAC221	*P. fusca*	*Coreopsis grandiflora*	Jinan, Shandong, 2002	DQ490748
HMLAC222	*P. fusca*	*Zinnia elegans*	Kumming, Yunnan, 2002	DQ490749
HMLAC225	*E. trifolii*	*Robinia pseudoacacia*	Laiyang, Shandong, 2003	DQ490769
HMLAC226	*E. polygoni*	*Polygonum aviculare*	Mengyin, Shandong, 2003	DQ490766
HMLAC227	*P. xanthii*	*Cucurbita pepo*	Laiyang, Shandong, 2003	DQ490750
HMLAC229	*Golovinomyces cichoracearum*	*Erigeron elongatus*	Mengyin, Shandong, 2003	DQ490751
JY1	*P. fusca*	*Conyza canadensis*	Yangling, Shaanxi, 2003	DQ490761
JY3	*A. mougeotii*	*L. chinense*	Yangling, Shaanxi, 2003	DQ490762
G2	*E. polygoni*	*Rumex patientia*	Budapest, Hungary, 2002	DQ490770
O1	*Phyllactinia guttata*	*Corylus avellana*	Canterbury, United Kingdom, 1999	DQ490771

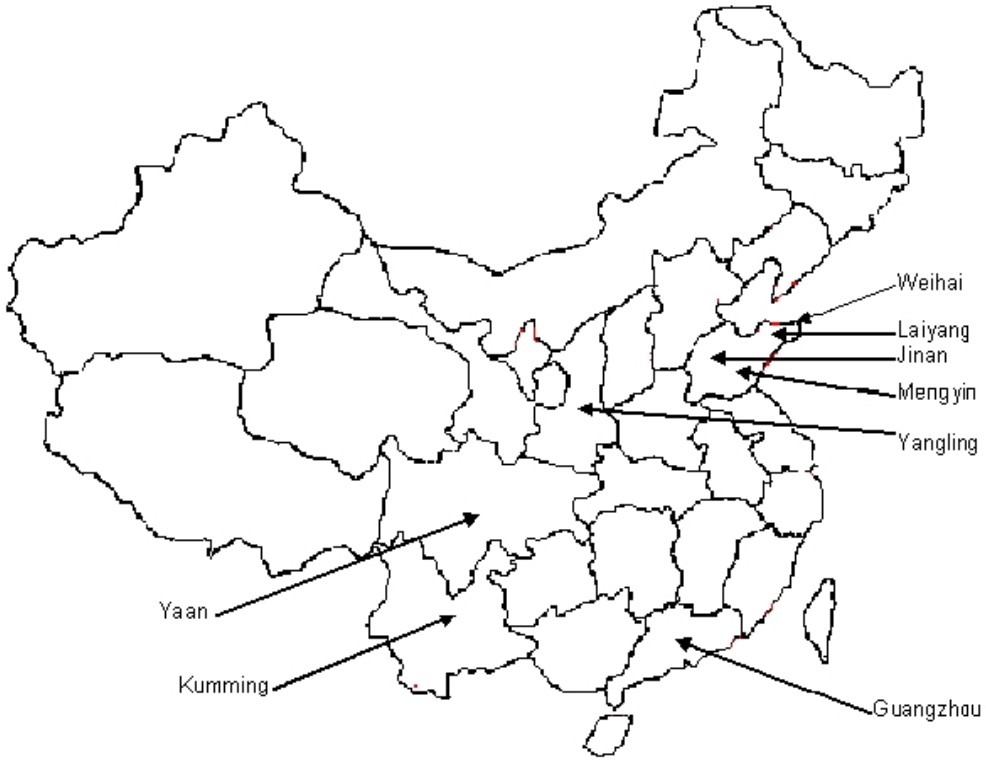

Fig. 1. Chinese locations where the *Ampelomyces* isolates included in this study came from.

Hungary, tobacco leaves infected with *Golovinomyces orontii*, and maintained *in vitro* (Szentiványi and Kiss, 2003), were inoculated with conidial suspensions of a total of nine isolates (HMLAC202-HMLAC204, HMLAC225-HMLAC227, HMLAC229, JY1 and JY3) and examined in a similar way. To fulfill Koch's postulates, the mycoparasites were re-isolated from the inoculated cucumber and tobacco leaves, respectively, using the isolation protocol described above. The six authentic *Ampelomyces* isolates (CBS 130.79, ATCC 201056, DSM 2222, MYA 3389, AQ10 and 263; Table 2), used in the morphological and cultural studies, were also included in these two tests as controls.

DNA extraction and PCR amplification and sequencing of the ITS region

Whole-cell DNA was extracted from 10-15 mg freeze-dried mycelia of the new isolates using a Qiagen DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The rDNA ITS

Table 2. Designations, host fungi, host plants, country and year of isolation and ITS sequence database accession numbers of the *Ampelomyces* isolates obtained in earlier studies and included in this work. The identity of the host fungi and host plants of the isolates were determined by their suppliers. The nomenclature of the *Erysiphaceae* follows the new system proposed by Braun and Takamatsu (2000) and Braun *et al*. (2002).

Isolate designation	Host fungus	Host plant	Country and year of isolation	ITS sequence database accession number	Reference
CBS 130.79	*Podosphaera xanthii*	*Cucurbita pepo*	Canada, 1975	U82449	Kiss and Nakasone, 1998
263	*Golovinomyces cichoracearum*	*Artemisia absinthium*	Canada, 1974	AF035782	Kiss and Nakasone, 1998
B55	*Podosphaera leucotricha*	*Malus domestica*	Hungary, 2002	AY663821	Szentiványi *et al*., 2005
MYA-3396	*P. leucotricha*	*M. domestica*	UK, 2002	AY663818	Szentiványi *et al*., 2005
MYA-3397	*P. leucotricha*	*M. domestica*	UK, 2002	AY663819	Szentiványi *et al*., 2005
MYA-3393	*P. leucotricha*	*M. domestica*	Germany, 2002	AY663820	Szentiványi *et al*., 2005
MYA-3389	*P. leucotricha*	*M. domestica*	Hungary, 1995	AY663815	Szentiványi *et al*., 2005
MYA-3390	*P. leucotricha*	*M. domestica*	Hungary, 2000	AY663816	Szentiványi *et al*., 2005
MYA-3395	*P. leucotricha*	*M. domestica*	Germany, 2002	AY663817	Szentiványi *et al*., 2005
U1	*P. pannosa*	*Prunus persica*	France, 1995	AY663822	Szentiványi *et al*., 2005
DSM 2222	*P. xanthii*	*Cucumis* sp.	Germany, ?*	U82450	Kiss and Nakasone, 1998
Ampelomyces sp.	*Sawadaea* sp.	*Acer macrophyllum*	USA, ?*	AY587139	Nischwitz et al., 2005
MYA-3399	*G. cichoracearum*	*Aster salignus*	UK, 1999	AY663823	Szentiványi *et al*., 2005
MYA-3400	*G. cichoracearum*	*A. salignus*	UK, 1999	AY663824	Szentiványi *et al*., 2005
AQ10	*Oidium* sp.	*Catha edulis*	Israel, ?*	AF035783	Kiss and Nakasone, 1998
ATCC201056	*Arthrocladiella mougeotii*	*Lycium halimifolium*	Hungary, 1990	AF035780	Kiss and Nakasone, 1998
CBS131.31	*G. cichoracearum*	*Helianthus tuberosus*	USA, 1931	AF035781	Kiss and Nakasone, 1998
ATCC200245	*E. penicillata*	*Platanus occidentalis*	USA, 1998	AF126817	Sullivan and White, 2000
AQ2	*E. penicillata*	*P. occidentalis*	USA, 1998	AF126818	Sullivan and White, 2000
DSM 2223	*E. sordida*	*Plantago* sp.	Germany, ?*	U82451	Kiss and Nakasone, 1998

* Missing data

region was amplified using the ITS1F/ITS4 fungal-specific primer pair (Gardes and Bruns, 1993) as described in Szentiványi *et al*. (2005). PCR products were purified using a QiaQuick PCR Purification Kit (Qiagen), cloned in the cloning vector pMD18-T (TaKaRa, Tokyo, Japan), and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer. Both strands were sequenced

with the primers used for PCR amplification of the ITS region. Electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer.

Sequence analyses

Multiple alignments of the analysed ITS sequences were obtained using MultAlin (Corpet, 1988). In addition to the newly determined ITS sequences, 20 sequences determined in earlier studies for different *Ampelomyces* isolates were included in the analyses (Table 2). The alignments were checked for ambiguous parts and edited using ProSeq 2.9 (Filatov, 2002). The PAUP* 4.0b10 software package (Swofford, 2003) was used to infer phylogenies. During preliminary analyses, the ITS sequence of a *Phoma* sp. (GenBank accession number AY663825) was chosen as an outgroup. Modeltest 3.06 (Posada and Crandall, 1998) was used to select the best-fit nucleotide substitution model based on Akaike information criterion (AIC, Akaike 1974). The best fit model was used in the maximum-likelihood (ML) analysis using heuristic search. Also the best fit model found by Modeltest was used in the distance-based neighbour-joining (NJ) analysis where the branches of the inferred tree were tested by bootstrap analysis (Felsenstein, 1985) with 5000 replicates. The inferred trees were visualized by TreeView 1.6.6 (Page, 1996). When calculating within- and between-group distances, the MEGA2.1 program (Kumar *et al*., 2001) with the p-distance model (Nei and Kumar, 2000) was used. Supporting materials are available upon request.

Results and discussion

New isolates and their mycoparasitic activity

A total of 27 isolates were obtained in this study (Table 1). These originated from pycnidia produced inside the cells of the conidiophores of different powdery mildew fungi collected from the field. Intracellular pycnidia were pyriform to globose, measured 36-123 × 22-45 µm, and contained unicellular, hyaline, mostly guttulate conidia, 4.2-7.5 × 2-3.6 µm. A detailed data set of morphological data for each isolate included in this study was reported by Liang (2004). According to Sutton (1980), these morphological patterns are characteristic of *Ampelomyces* mycoparasites. Twenty-three of the new isolates came from different parts of China (Fig. 1) while four of them were obtained from Europe. All of them were characterized by a radial growth rate of only 0.05-0.88 mm/day on PDA at room temperature. In earlier works (for a review, see Kiss *et al*., 2004), two types of growth were distinguished

among isolates identified as *'Ampelomyces'*: the faster-growing isolates extended at 3-4 mm radial growth/day in culture at room temperature, while this value was only 0.1-1 mm/day for the slower-growing isolates. All the 27 isolates obtained in this study, similar to the six authentic *Ampelomyces* isolates, belonged to this latter group.

The mycoparasitic tests revealed that all the new isolates, as well as all the six authentic *Ampelomyces* isolates, produced intracellular pycnidia in the conidiophores of *P. xanthii* and/or *G. orontii* when these powdery mildew species were inoculated with conidial suspensions of the isolates. Re-isolation of the mycoparasites was always successful from the inoculated powdery mildew colonies. These tests, together with the morphological and cultural characteristics of the new isolates, confirmed that the new isolates can be identified as *Ampelomyces* mycoparasites and they were not confused with other pycnidial mycoparasites of powdery mildew fungi which do not produce pycnidia inside the powdery mildew mycelia (Sullivan and White, 2000) and are characterized by a faster growth rate in culture (Kiss *et al*., 2004).

Phylogenetic analysis

The ITS sequences of the 27 new *Ampelomyces* isolates were analyzed together with 20 sequences of other *Ampelomyces* isolates obtained in earlier studies (Table 2). During the analysis, a 422 characters long alignment was used for inferring phylogenies. The GTR+G model was selected as the best fit model. The base frequencies (A, C, G, T) were 0.2507, 0.2235, 0.2088 and 0.3170, respectively. The values of the rate matrix (rAC, rAG, rAT, rCG, rCT, rGT) were 3.0896, 4.3682, 1.5209, 1.4538, 9.2549 and 1.0000, respectively, and the Gamma shape parameter was 0.4188. Both the ML and NJ analyses resulted in the same clustering of the strains. The ML tree is shown in Fig. 2. The sequences grouped into seven main clades and all but two contained isolates originating from China. Although the clustering of the seven clades was unambiguous in both ML and NJ analyses, the relative branching order of these groups was not supported by high bootstrap values.

As shown in Fig. 2, the 23 *Ampelomyces* isolates coming from China were genetically diverse based on their ITS sequences. Those included in clades 3, 4, 5 and 7 showed close phylogenetic relationships with European and/or North American isolates sequenced in previous studies, while a total of 16 isolates obtained in three provinces of China formed a distinct group (Clade 1). Most Chinese isolates, 16 out of 23, were obtained in Shandong province (Table 1), but these clustered in four different groups (Clades 1, 3, 4 and 7). Similarly, the two isolates coming from Shaanxi province belonged to two

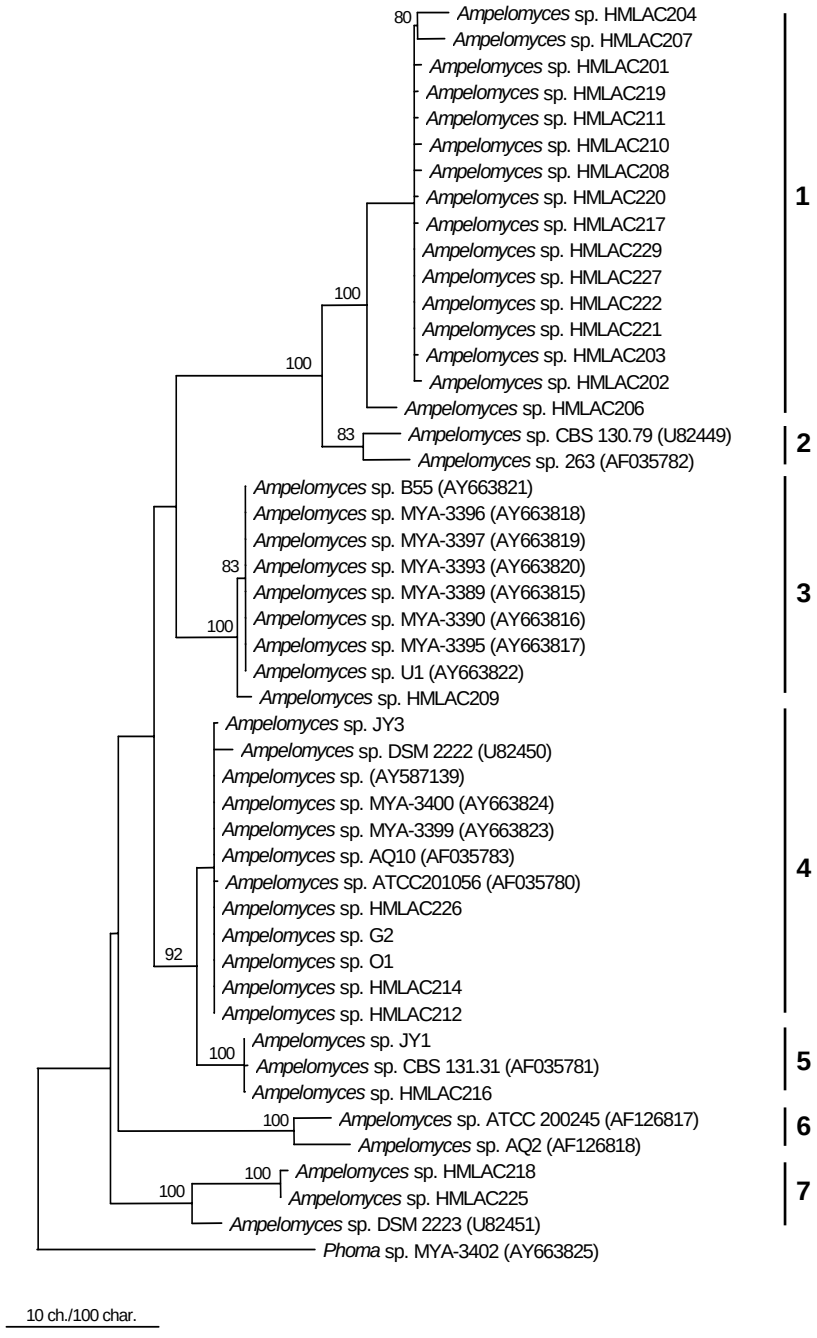
80
Ampelomyces sp. HMLAC204
Ampelomyces sp. HMLAC207
Ampelomyces sp. HMLAC201
Ampelomyces sp. HMLAC219
Ampelomyces sp. HMLAC211
Ampelomyces sp. HMLAC210
Ampelomyces sp. HMLAC208
Ampelomyces sp. HMLAC220
Ampelomyces sp. HMLAC217
Ampelomyces sp. HMLAC229
Ampelomyces sp. HMLAC227
100
Ampelomyces sp. HMLAC222
Ampelomyces sp. HMLAC221
Ampelomyces sp. HMLAC203
100
Ampelomyces sp. HMLAC202
Ampelomyces sp. HMLAC206
1
83
Ampelomyces sp. CBS 130.79 (U82449)
Ampelomyces sp. 263 (AF035782)
2
Ampelomyces sp. B55 (AY663821)
Ampelomyces sp. MYA-3396 (AY663818)
Ampelomyces sp. MYA-3397 (AY663819)
83
Ampelomyces sp. MYA-3393 (AY663820)
Ampelomyces sp. MYA-3389 (AY663815)
100
Ampelomyces sp. MYA-3390 (AY663816)
Ampelomyces sp. MYA-3395 (AY663817)
Ampelomyces sp. U1 (AY663822)
3
Ampelomyces sp. HMLAC209
Ampelomyces sp. JY3
Ampelomyces sp. DSM 2222 (U82450)
Ampelomyces sp. (AY587139)
Ampelomyces sp. MYA-3400 (AY663824)
Ampelomyces sp. MYA-3399 (AY663823)
Ampelomyces sp. AQ10 (AF035783)
Ampelomyces sp. ATCC201056 (AF035780)
Ampelomyces sp. HMLAC226
Ampelomyces sp. G2
92
Ampelomyces sp. O1
Ampelomyces sp. HMLAC214
Ampelomyces sp. HMLAC212
4
Ampelomyces sp. JY1
100
Ampelomyces sp. CBS 131.31 (AF035781)
Ampelomyces sp. HMLAC216
5
100
Ampelomyces sp. ATCC 200245 (AF126817)
Ampelomyces sp. AQ2 (AF126818)
6
100
Ampelomyces sp. HMLAC218
100
Ampelomyces sp. HMLAC225
Ampelomyces sp. DSM 2223 (U82451)
7
Phoma sp. MYA-3402 (AY663825)
10 ch./100 char.

Fig. 2. The maximum-likelihood tree of 47 *Ampelomyces* sequences, 27 of them determined in the present study (Table 1), and 20 from previous studies (Table 2), with a *Phoma* sequence as outgroup as inferred with PAUP* 4.0b10 program package (Swofford, 2003). GenBank accession numbers of the sequences determined in earlier studies are shown in brackets and those of the newly determined sequences are included in Table 1. The gaps of the 422 characters long alignment were handled as missing characters. The GTR+G model was used as the best-fit model based on the AIC results of program Modeltest 3.06 (Posada and Crandall 1998). The base frequencies (A, C, G, T) were 0.2507, 0.2235, 0.2088 and 0.3170, respectively; the values of the rate matrix (rAC, rAG, rAT, rCG, rCT, rGT) were 3.0896, 4.3682, 1.5209, 1.4538, 9.2549 and 1.0000, respectively ; the shape parameter was 0.4188. The bootstrap values were obtained from the neighbour-joining analyses. The values shown above the branches are percentages of 5000 replicates, and the scores below 75% are not shown. The seven clades (1-7) discussed in the text are indicated on the tree. Bar = 10 changes on 100 character.

Clades, 4 and 5, and the three isolates obtained in Sichuan Province were also included in two Clades, 1 and 7, respectively.

Thus, no evidence of local populations of *Ampelomyces* mycoparasites with similar ITS sequences was found. In contrast, the ITS sequences of some isolates obtained in China were identical with those of some European and/or North American isolates determined in previous studies (Table 2). For example, the ITS sequence of isolate HMLAC226 from Shandong was identical with that of a number of other isolates (MYA-3399, AQ10, G1, O1, HMLAC214, etc.) obtained in Hungary, United Kingdom, Germany, USA and Israel. All these sequences clustered together in clade 4. Similarly, the ITS sequences of isolates JY1 and HMLAC216, isolated in Shaanxi and Guangdong, respectively, were identical with that of isolate CBS 131.31 obtained in the USA in 1931. These three isolates were included in clade 5. These data suggest a global distribution of at least some of the *Ampelomyces* mycoparasites with identical ITS sequences.

Apparently, the clustering of the *Ampelomyces* isolates obtained in China has suggested some correlation with the powdery mildew and host plant species where these mycoparasites came from. Ten out of the 23 isolates were obtained from asteraceous host plants infected with *Podosphaera fusca* (Table 1) and all but one grouped together in Clade 1 (Fig. 2). In addition, the four newly obtained isolates coming from cucurbitaceous hosts infected with *P. xanthii* (HMLAC207, HMLAC210, HMLAC217 and HMLAC227) were also included in clade 1. However, isolate JY1 obtained from *P. fusca* was included in Clade 5, and isolates HMLAC202, HMLAC204 and HMLAC229, coming from mycohosts other than *Podosphaera* (Table 1), were also included in clade 1. These data suggest that the *Ampelomyces* isolates clustered in clade 1 should not be regarded as a group of mycoparasites specialized to two *Podosphaera* species infecting asteraceous and cucurbitaceous plants, respectively. In

addition, the identity of *Podosphaera* spp., infecting various species of the *Asteraceae* and the *Cucurbitaceae*, is controversial (Braun and Takamatsu, 2000) and these powdery mildew fungi are genetically diverse (Hirata *et al*., 2000). Thus, the clustering of various isolates does not suggest any correlation with either the powdery mildew and host plant species where these mycoparasites came from or their geographical origin.

Clearly, the number of the newly obtained *Ampelomyces* isolates was not enough for a meaningful analysis of their distribution in different fungal and plant hosts. The data obtained showed that, on the one hand, *Ampelomyces* mycoparasites with similar or identical ITS sequences were found in many different powdery mildew hosts (see Clades 1, 4, 5 and 7), and, on the other hand, mycoparasites with different ITS sequences occur in the same powdery mildew species. Isolates HMLAC202 and JY3, for example, were included in Clades 1 and 4, respectively, although both came from *Arthrocladiella mougeotii* infecting *Lycium chinense* in China.

Curiously, isolate HMLAC209 was closely related to a number of isolates obtained from apple and peach powdery mildew in Europe (see Clade 3). Szentiványi *et al*. (2005) have recently suggested that *Ampelomyces* mycoparasites in these two powdery mildew species might be isolated in time from the rest of the *Ampelomyces* populations in a given environment as apple and peach powdery mildew start their life cycle early in the season while most species of the *Erysiphaceae* start to sporulate and spread in the same environment only later in the season. The close phylogenetic relationship between isolate HMLAC209, obtained from *P. ferruginea* infecting *Sanguisorba officinalis* in Shandong, and this group of European isolates requires further analysis.

The distances among ITS sequences of *Ampelomyces* isolates were calculated both within- and between the seven clades. The values between the seven clades were generally much higher than the distances within the clades (Table 3). The three highest within-clade distances characterized Clades 2, 6 and 7, respectively, which included two or three isolates only. All between-clade distances were greater than the highest within-clade distance except the distance between Clades 4 and 5 which included isolates with less diverse ITS sequences.

An earlier study reporting sequence divergence values higher than 10-15% between some ‘true’ *Ampelomyces* isolates (Kiss and Nakasone, 1998) is in agreement with these results. Sullivan and White (2000), Nischwitz *et al*. (2005) and Szentiványi *et al*. (2005) have also shown that ITS sequences were diverse in *Ampelomyces*. This could mean that the genus, considered as monotypic by some authors, consists of more than one species as suggested by

Table 3. The distances of ITS sequences within and between the groups of the studied *Ampelomyces* sequences. Each group corresponds to a clade as defined in Figure 2.

	Between						**Within**
Group	**1**	**2**	**3**	**4**	**5**	**6**	
1							0.015
2	0.090						0.048*
3	0.148	0.124					0.003
4	0.144	0.134	0.083				0.003
5	0.151	0.135	0.092	0.041			0.002
6	0.166	0.160	0.147	0.139	0.140		0.053*
7	0.167	0.161	0.130	0.114	0.122	0.151	0.045

* only two sequences in the group

Kiss and Nakasone (1998). Although both inter- and intra-specific divergences in ITS sequences are variable in various fungal taxa and thus cannot serve as reliable bases for species delineation (e.g., Seifert, 1995; Taylor *et al.*, 2000), the sequence divergence values reported in this study (Table 3), and also in earlier works, clearly showed that a taxonomic revision of the genus *Ampelomyces* is warranted.

In conclusion, *Ampelomyces* mycoparasites isolated from different powdery mildew fungi in China proved to be genetically diverse based on the analysis of the nrDNA ITS sequences. The diversity in their ITS sequences might suggest that they represent distinct species of *Ampelomyces*. The ITS sequences of some isolates obtained in China were identical to those of some European and/or North American isolates (see clades 4 and 5) which indicates a global distribution of these mycoparasites. At the same time, 16 Chinese isolates formed a distinct group (clade 1) which was only distantly related to the already known groups of the European and the North American *Ampelomyces* isolates. Current results suggest that there are not much local differentiations (adaptations) of *Ampelomyces* mycoparasites with regard to their hosts as well as to geographical regions, with an exception of apple powdery mildew (Szentiványi *et al.*, 2005). The genetically different *Ampelomyces* isolates obtained in China will be tested as biocontrol agents of powdery mildew diseases of economically important crops, particularly to determine whether there are some degrees of quantitative associations between isolates and their host fungi/plants.

Acknowledgements

This work was supported by an EU-INCO project (SMADIA ICA4-2000-10011). The support of the János Bolyai Research Fellowship, awarded to GMK and LK, is also gratefully acknowledged. GMK is a postdoctoral research fellow of the Hungarian Research Fund (OTKA, D048333).

References

Akaike, H. (1974). A new look at the statistical model identification. IEEE Transactions on Automatic Control 19: 716-723.

Bélanger, R.R. and Labbé, C. (2002). Control of powdery mildews without chemicals: prophylactic and biological alternatives for horticultural crops. In: *The Powdery Mildews*: *A Comprehensive Treatise* (eds. R.R. Bélanger, W.R. Bushnell, A.J. Dik and T.L.W. Carver). APS Press, St. Paul, USA: 256-267.

Belsare, S.W., Moniz, L. and Deo, V.B. (1980). The hyperparasite *Ampelomyces quisqualis* Ces. from Maharashtra State, India. Biovigyanam 6: 173-176.

Braun, U. and Takamatsu, S. (2000). Phylogeny of *Erysiphe*, *Microsphaera*, *Uncinula* (Erysipheae) and *Cystotheca*, *Podosphaera*, *Sphaerotheca* (Cystotheceae) inferred from rDNA ITS sequences – some taxonomic consequences. Schlechtendalia 4: 1-33.

Braun, U., Cook, R.T.A., Inman, A.J. and Shin, H.D. (2002). The taxonomy of the powdery mildew fungi. In: *The Powdery Mildews*: *a comprehensive treatise* (eds. R.R. Bélanger, W.R. Bushnell, A.J. Dik and T.L.W. Carver). APS Press, St. Paul, USA: 13-55.

Chen, D.R. and Yang, Z.S. (1990). Studies on the hyperparasite of powdery mildew. Journal of Nanjing Agricultural University 13: 64-68.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. Nucleic Acids Research 16: 10881-10890.

De Bary, A. (1870). *Eurotium*, *Erysiphe*, *Cicinnobolus*, nebst Bemerkungen über die Geschlechtsorgane der Ascomyceten. In: *Beiträge zur Morphologie und Physiologie der Pilze* (eds. A. De Bary and M. Woronin). Verlag von C. Winter, Frankfurt a. M., Germany: 1-95.

Falk, S.P., Gadoury, D.M., Cortesi, P., Pearson, R.C. and Seem, R.C. (1995). Parasitism of *Uncinula necator* cleistothecia by the mycoparasite *Ampelomyces quisqualis*. Phytopathology 85: 794-800.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

Filatov, D.A. (2002). ProSeq: A software for preparation and evolutionary analysis of DNA sequence data sets. Molecular Ecology Notes 2: 621-624.

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specifity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Goh, T.K. (1999). Single-spore isolation using a hand-made glass needle. Fungal Diversity 2: 47-63.

Hashioka, Y. and Nakai, Y. (1980). Ultrastructure of pycnidial development and mycoparasitism of *Ampelomyces quisqualis* parasitic on Erysiphales. Transactions of the Mycological Society of Japan 21: 329-338.

Hino, I. and Kato, H. (1929). Cicinnoboli parasitic on mildew fungi. Bulletin of the Miyazaki Collegium of Agriculture and Forestry 1: 91-98.

Hirata, T., Cunnington, J.H., Paksiri, U., Limkaisang, S., Shishkoff, N., Grigaliunaite, B., Sato, Y. and Takamatsu, S. (2000). Evolutionary analysis of subsection *Magnicellulatae* of *Podosphaera* section *Sphaerotheca* (Erysiphales) based on the rDNA internal transcribed spacer sequences with special reference to host plants. Canadian Journal of Botany 78: 1521-1530.

Hofstein, R., Daoust, R.A. and Aeschlimann, J.P. (1996). Constrains to the development of biofungicides: the example of "AQ10", a new product for controlling powdery mildews. Entomophaga 41: 455-460.

Kiss, L. (1997). Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi inferred from RFLP analysis of the rDNA ITS region. Mycological Research 101: 1073-1080.

Kiss, L. (1998). Natural occurrence of *Ampelomyces* intracellular mycoparasites in mycelia of powdery mildew fungi. New Phytologist 140: 709-714.

Kiss, L. (2003). Fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Management Science 59: 475-483.

Kiss, L. and Nakasone, K.K. (1998). Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi. Current Genetics 33: 362-367.

Kiss, L., Russell, J., Szentiványi, O., Xu, X. and Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonists of powdery mildew fungi. Biocontrol Science and Technology 14: 635-651.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001). MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17: 1244–1245.

Lee, S.Y. (1999). *Biological control of cucumber powdery mildew using a hyperparasite, Ampelomyces quisqualis*. PhD thesis. Chungnam National University, Taejon, Korea. [In Korean.]

Lee, S.Y., Lee, S.B. and Kim, C.H. (2004). Biological control of powdery mildew by Q-fect WP (*Ampelomyces quisqualis* 94013) in various crops. IOBC/wprs Bulletin 27: 329-331.

Liang, C. (2004). *Population diversity, biological characters and biocontrol potential of the genus Ampelomyces*. Ph.D. thesis. Shenyang Agricultural University, People's Republic of China. [In Chinese.]

Liang, C., Zhao, H.H., Li, B.D., Zhang, Y.H. and Lu, G.Z. (2004). Biological characteristics of *Ampelomyces quisqualis* hyperparasite on tickseed powdery mildew. Journal of Yunnan Agricutural University 19: 648-652. [In Chinese.]

Mhaskar, D.N. and Rao, V.G. (1974). The mycoparasite *Ampelomyces quisqualis* Ces. in artificial culture II. Effect of environmental factors. Phytopathologia Mediterranea 13: 147-154.

Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York, USA.Nischwitz, C., Newcombe, G. and Anderson, C.L. (2005). Host specialization of the mycoparasite *Eudarluca caricis* and its evolutionary relationship to *Ampelomyces*. Mycological Research 109: 421-428.

Page, R.D.M. (1996). TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.

Paulitz, T.C. and Bélanger R.R. (2001). Biological control in greenhouse systems. Annual Review of Phytopathology 39: 103-133.

Philipp, W.D. and Crüger, G. (1979). Parasitismus von *Ampelomyces quisqualis* auf Echten Mehltaupilzen an Gurken und anderen Gemüsearten. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 86: 129-142.

Posada, D. and Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Rudakov, O.L. (1979). Fungi of the genus *Ampelomyces* Ces. Ex Schlecht. Mikologiya i Fitopatologiya 13: 104-110. [In Russian.]

Seifert, K.A., Wingfield, B.D. and Wingfield, M.J. (1995). A critique of DNA sequence analysis in the taxonomy of filamentous Ascomycetes and ascomycetous anamorphs. Canadian Journal of Botany 73 (Suppl. 1): S760-S767.

Shin, H-D. (1994). Isolation and identification of hyperparasites against powdery mildew fungi in Korea. Korean Journal of Mycology 22: 355-365. [In Korean.]

Shin, H-D. and Kyeung, H-Y. (1994). Isolation of hyperparasitic fungi to powdery mildews and selection of superior isolates for biocontrol of cucumber powdery mildew. RDA Journal of Agricultural Science 36: 141-151.

Speer, E.O. (1978). Beitrag zur Morphologie von *Ampelomyces quisqualis* Ces. Sydowia 31: 242-246.

Sullivan, R.F. and White, J.F. Jr. (2000). *Phoma glomerata* as a mycoparasite of powdery mildew. Applied and Environmental Microbiology 66: 425-427.

Sundheim, L. (1982). Control of cucumber powdery mildew by the hyperparasite *Ampelomyces quisqualis* and fungicides. Plant Pathology 31: 209-214.

Sundheim, L. and Krekling, T. (1982). Host-parasite relationships of the hyperparasite *Ampelomyces quisqualis* and its powdery mildew host *Sphaerotheca fuliginea*. Journal of Phytopathology 104: 202-210.

Sutton, B.C. (1980). *The Coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata*. Commonwealth Mycological Institute, Kew, UK.

Swofford, D.L. (2003). PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Szentiványi, O. and Kiss, L. (2003). Overwintering of *Ampelomyces* mycoparasites on apple trees and other plants infected with powdery mildews. Plant Pathology 52**:** 737-746.

Szentiványi, O., Kiss, L., Russell, J.C., Kovács, G.M., Varga, K., Jankovics, T., Lesemann, S., Xu, X.M. and Jeffries, P. (2005). *Ampelomyces* mycoparasites from apple powdery mildew identified as a distinct group based on single-stranded conformation polymorphism analysis of the rDNA ITS region. Mycological Research 109: 429-438.

Sztejnberg, A., Galper, S., Mazar, S. and Lisker, N. (1989). *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. Journal of Phytopathology 124: 285-295.

Tai, F.L. (1979). *Sylloge Fungorum Sinica*. Science Press, Peking, China.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S. and Fisher, M.C. (2000). Phylogenetic species concept recognition and species concepts in fungi. Fungal Genetics and Biology 31: 21-32.

Tsay, J.G. and Tung, B. (1991). *Ampelomyces quisqualis* Ces. Ex Schlecht., a hyperparasite of the asparagus bean powdery mildew pathogen *Erysiphe polygoni* in Taiwan. Transactions of the Mycological Society of R.O.C. 6: 55-58.

Whipps, J.M. and Lumsden, R.D. (2001). Commercial use of fungi as plant disease biological control agents: status and prospects. In: *Fungi as Biocontrol Agents*: *Progress, Problems and Potential* (eds. T.M. Butt, C. Jackson and N. Magan). CABI Publishing, Wallingford, UK: 9-22.

(Received 20 December 2005; accepted 20 September 2006)