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Ribosomal DNA internal transcribed spacer sequences do not support the species status of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew fungi

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Abstract Phylogenetic relationships among *Ampelomyces* isolates, pycnidial hyperparasites and biological control agents of powdery mildews, were inferred from internal transcribed spacer (ITS) sequences of the ribosoma1 DNA (rDNA). Currently, these hyperparasites are considered to be a single species, *A. quisqualis,* despite observed morphological and cultural differences. Ten *Ampelomyces* isolates, representing seven previously defined ITS RFLP groups, were sequenced and analyzed. Sequence-divergence values among isolates belonging to different RFLP groups ranged from 4.3 to 22.4%, suggesting that these isolates may represent different taxa. When *Ampelomyces* ITS sequences were analyzed by cladistic methods with the sequences of other ascomycetous fungi, they formed two lineages in the Dothideales. Slow-growing *Ampelomyces* isolates formed a clade with *Leptosphaeria microscopica* and *L. nodorum,* whereas fast-growing *Ampelomyces* isolates formed a clade with *Epicoccum nigrum.* Sequence-divergence values between these two clades ranged from 17.3 to 22.4%, suggesting that the taxa in the two clades are not closely related and possibly not congeneric. The data presented here indicate that the identification of *'A. quisqualis'* isolates used in biological control experiments should be re-evaluated.

Key words *Ampelomyces* spp. · Biological control · Powdery mildews · Ribosomal DNA Internal transcribed spacer (ITS) region

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Introduction

The interactions between powdery mildew fungi, biotrophic parasites of many plants, and their most common and widespread intracellular hyperparasites, belonging to the coelomycete genus *Ampelomyces,* were the first thoroughly studied interfungal parasitic relationships in nature (De Bary 1870). *Ampelomyces* hyperparasites penetrate into the hyphae of powdery mildews, continue their growth internally and produce pycnidia in one or two cells of the hyphae, conidiophores or conidium initials and occasionally in young, immature cleistothecia of their fungal hosts (e.g. Speer 1978; Philipp and Crüger 1979; Kranz 1981; Falk et al. 1995a, b). Therefore, to a certain extent these hyperparasites suppress asexual and sexual sporulation of, and may eventually kill, the powdery mildew colonies. More than 65 species of the Erysiphaceae representing nine genera are known to be natural hosts of *Ampelomyces* (Falk et al. 1995a; Kiss 1997b). Experiments have shown that an *Ampelomyces* hyperparasite found in a given species of powdery mildew fungi is not specialized to it and is able to infect many other species of the Erysiphaceae (e.g. De Bary 1870; Philipp and Crüger 1979; Sztejnberg et al. 1989; Falk et al. 1995b; Kiss and Vajna 1995). No sexual stage of these hyperparasites is known (Philipp and Crüger 1979; Falk et al. 1995a).

The use of these hyperparasites in biological control experiments of many crops infected with powdery mildews have shown promising results both in the field and greenhouse (Jarvis and Slingsby 1977; Sundheim and Tronsmo 1988; Sztejnberg et al. 1989; Philipp et al. 1990; Marboutie et al. 1995; Falk et al. 1995 a, b). At present, an *Ampelomyces* isolate is the only registered biocontrol agent of powdery mildews, commercialized as 'AQ10 Biofungicide' by Ecogen Incorporated and used mainly against grape powdery mildew as part of an integrated pest-management program (Hofstein and Fridlender 1994).

There are more than 40 *Ampelomyces* species validly described in the older literature; however, most of these binomials are not currently used, and the genus is in need of

Table 1 *Ampelomyces* isolates used in this study

Isolate	Host fungus/ plant of origin	Country of origin	Type of growth	RFLP group[a]	Source[b]
Ampelomyces sp. ATCC 201059	*Erysiphe cichoracearum* on *Cucurbita pepo*	Hungary	Fast-growing	I	PPI
A. quercinus CBS 633.92 (ATCC 36786)	*Microsphaera alphitoides* on *Quercus* sp.	Russia	Fast-growing	II	CBS
A. humuli ATCC 38616	*Sphaerotheca macularis* on ...[c]	Russia	Fast-growing	II	ATCC
A. quisqualis CBS 130.79	*S. fuliginea* on *C. pepo*	Canada	Slow-growing	III	CBS
A. quisqualis DSM 2222	*E. cichoracearum* on *Cucumis* sp.	Germany	Slow-growing	IV	DSM
Ampelomyces sp. ATCC 201056	*Arthrocladiella mougeotii* on *Lycium halimifolium*	Hungary	Slow-growing	IV	PPI
A. quisqualis DSM 2223	*E. sordida* on *Plantago* sp.	Germany	Slow-growing	V	DSM
A. quisqualis CBS 131.31	*E. cichoracearum* on *Helianthus tuberosus*	USA	Slow-growing	VI	CBS
A. quisqualis 263	*E. cichoracearum* on *Artemisia absinthium*	Canada	Slow-growing	VII	W. R. Jarvis (AC)
A. quisqualis AQ10	No data	Israel	Slow-growing	No data	Ecogen Inc

[a]See Kiss (1997a)
[b]AC = Agriculture Canada, Greenhouse and Processing Crops Research Centre, Harrow, Canada; ATCC = American Type Culture Collection, Rockville, Maryland; CBS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; PPI = Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary
[c]Missing data

taxonomic revision (Rudakov 1979; Sutton 1980; Kranz 1981). For the past 15-20 years, the genus has generally been considered monotypic and represented by a single species, *A. quisqualis.* Most biological control experiments were based on this single-species concept, although morphological and cultural differences among various *Ampelomyces* isolates have been reported (Mhaskar 1974; Mhaskar and Rao 1974; Rudakov 1979; Belsare et al. 1980; Sz. Nagy and Vajna 1990; Kiss and Vajna 1995).

Recently, RFLP analysis of the ITS region of nuclear rDNA showed high genetic diversity in a worldwide sampling of 46 *Ampelomyces* isolates (Kiss 1997a). Seven RFLP groups were identified among these isolates which could be grouped in two clades, according to the growth rate of isolates in culture. These results suggest that, contrary to the generally accepted status, the binomial *'A. quisqualis'* should be regarded as the name of a problematic species complex. Precise classical and molecular methods are needed to identify the different isolates of *'A. quisqualis'* employed as biocontrol agents of powdery mildews.

The objectives of the present study were: (1) to analyze the phylogenetic relationships among *Ampelomyces* isolates representing different, previously defined RFLP groups (Kiss 1997a), and (2) to infer the phylogenetic relationships of these asexual fungi among the ascomycetes, according to the holomorph concept (Reynolds and Taylor 1993; Hawksworth 1994; Taylor 1995). To achieve these objectives, sequence data of the rDNA ITS region of ten *Ampelomyces* isolates were determined and compared with the corresponding sequences of other ascomycetous fungal species available from GenBank and EMBL databases.

Materials and methods

Isolates and culture conditions. The isolate designation, host fungi and plants, country of origin, type of growth, RFLP group and the source of the ten *Ampelomyces* isolates included in this study are listed in Table 1. Isolates were maintained at room temperature on Czapek-Dox agar supplemented with 2% malt extract (MCzA). Radial growth was determined daily by measuring the diameter of colonies inoculated as mycelial discs of IO-mm diameter on MCzA at 23°C.

DNA extraction and amplification and sequencing of the ITS region. The protocol for DNA extraction and PCR amplification of the rDNA ITS region from *Ampelomyces* mycelia is described in Kiss (1997a). Additionally, DNA of some isolates was extracted using a modified protocol described by Cenis (1992). This crude total DNA preparation was then further purified with a USBioclean kit (USB, Cleveland, Ohio). The ITS region, which includes the internal transcribed spacer regions 1 and 2, the 5.8 S rRNA gene, and the flanking 18 S and 26 S genes, was amplified with primers ITS5 and ITS4 (White et al. 1990) in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) or PTC 200 DNA Engine (MJ Research, Watertown, Mass.). The cycling parameters employed are described in Kiss (1997a) or, alternatively, were as follows: an initial 93°C denaturation for 2 min, followed by 35 cycles of primer annealing for 2 min

Table 2 Sources of sequence data

Fungi	Source	EMBL/ GenBank accession no.
Alternaria alternata	Jasalavich et al. 1995	U05195
Ampelomyces quercinus		
CBS 633.92 (ATCC 36786)	This paper	AF035778
Ampelomyces humuli		
ATCC 38616	This paper	AF035779
Ampelomyces spp.		
ATCC 201059	This paper	U82452
CBS 130.79	This paper	U82449
DSM 2222	This paper	U82450
ATCC 201056	This paper	AF035780
DSM 2223	This paper	U82451
CBS 131.31	This paper	AF035781
263	This paper	AF035782
AQ10	This paper	AF035783
Epicoccum nigrum	Rollo et al. 1995	–
Leptosphaeria bicolor	Morales et al. 1995	U04203
L. doliolum	Morales et al. 1995	U04207
L. maculans Leroy	Morales et al. 1993	M96384
L. maculans Unity	Morales et al. 1993	M96383
L. microscopica	Morales et al. 1995	U04234
L. nodorum	Morales et al. 1995	U04237

at 53°C, extension for 2 min at 72°C, and a denaturation step of 1 min at 93°C. PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was completed by the Advanced Genetic Analysis Center (St. Paul, Minn.) or Génome Express (Grenoble, France) using primers ITS1, ITS2, ITS 3 or ITS4 (White et al. 1990). The sequences have been deposited in GenBank (see Table 2).

Sequence analysis. Preliminary comparisons of ITS sequences of *Ampelomyces* isolates with the corresponding sequences of all ascomycetous fungi available from GenBank and EMBL databases were performed using UPGMA clustering (Rohlf 1993). Subsequently, detailed analyses included only ITS sequences of the closely related fungi listed in Table 2. These sequences were entered on MacClade, version 3.1 (Maddison and Maddison 1992), and aligned manually. Sequence alignments are available from the corresponding author on request. When ambiguity in alignment was encountered, the alignment that produced the fewest number of informative sites was preferred. PAUP, version 3.1.1 (Swofford 1993) was employed to obtain base-line maximum parsimony trees from unordered nucleotide data (Fitch 1971). Heuristic tree searches with the TBR (tree bisection and reconnection) branch swapping and ACCTRAN (accelerated transformation) optimization options were selected. The consistency index (CI, Kluge and Farris 1969) and retention index (RI, Farris 1989), excluding uninformative characters, are reported for base-line trees. The effect of weighting transversions 1.1.-, 2-, and 3-times more than transitions was evaluated. Different alignments were analyzed to accommodate ambiguous areas and insertions/deletions. Support for monophyletic clades was estimated with decay (Bremer 1988; Donoghue et al. 1992) and bootstrap analyses (Felsenstein 1985). Although bootstrap (Sanderson 1989) and decay analyses have limitations, they do indicate relative support for the clades. Bootstrap analyses were implemented using 1000 replicates of heuristic searches.

Results

Three of the ten *Ampelomyces* isolates sequenced (ATCC 201059, CBS 633.92 and ATCC 38616) were fast-growing isolates characterized by 3–4-mm radial growth per day on MCzA at 23°C. The other isolates sequenced were slow-growing (Table 2) with a radial growth of only 0.1-1 mm/day. The cultural characteristics of slow-growing and fast-growing isolates were extremely different. The isolates sequenced, except for the registered AQ10 isolate, were chosen based on a previous RFLP analysis of their ITS region that showed the existence of seven RFLP groups in a worldwide collection (Kiss 1997a). At least one isolate of each RFLP group was included in this study.

A single product was amplified from the isolates. The fast-growing isolates produced a fragment that was slightly shorter (about 5.50 bp) than the fragment amplified by the slow-growing isolates (about 600 bp).

UPGMA clustering analyses of ITS sequences of all ascomycetous fungi available in the GenBank and EMBL databases suggested that the *Ampelomyces* isolates studied were closely related to some *Leptosphaeria* spp., *Alternaria alternata* and *Epicoccum nigrum* (data not shown). In subsequent cladistic analyses, only these closely related taxa were included because of difficulty in sequence alignment. These taxa are listed in Table 2. *Leptosphaeria bicolor* was chosen as the outgroup taxon based on these initial analyses, because it appeared to be somewhat related to the *Ampelomyces* isolates and its sequences could be aligned without too much difficulty with the ingroup taxa.

Sequences of the ITS region of the slow- and fast-growing *Ampelomyces* isolates as well as *L. microscopica, L. nodorum* and *E. nigrum* were relatively easy to align. However, alignment of *A. alternata, L. maculans, L. doliolum* and *L. bicolor* were more problematical, especially the first 80 bp at the 5′-end. The sequence-divergence values ranged from 0.4 to 6.0% among the three fast-growing *Ampelomyces* isolates and *E. nigrum,* and from 0.2 to 19.5% among the seven slow-growing isolates (Table 3). The lowest divergence values among the sequences occurred between fast-growing isolates CBS 633.92 and ATCC 38616 (0.4%), and among the slow-growing isolates AQ10,DSM2222 and ATCC201056 (0.2–0.8%). The fast-growing isolates appear to be fairly homogenous whereas the slow-growing group is more complex and can be broken down into four subgroups: (1) CBS 130.79 and 263 (sequence divergence 6.4%) (2) AQ10, DSM 2222, and ATCC 201056 (sequence divergence 0.2–0.8%), (3) CBS 131.31 and (4) DSM 2223. Sequence-divergence values between the fast-growing and slow-growing groups ranged from 17.1 to 22.4%.

In the first cladistic analysis, gaps were coded as missing data, and the data were treated as unordered. There were 187 potentially informative nucleotide positions. Three most-parsimonious trees, 679 steps long with a CI, excluding un-informative characters, of 0.570 and a RI of 0.662, were recovered. The three trees differed only in the pairing of the three slow-growing isolates, AQ10, DSM 2222 and ATCC 201056. In the phylogram presented in Fig. 1, these three isolates are shown as an unresolved trichotomy. Figure 1 shows one of the most-parsimonious

Table 3 Sequence divergence values - mean distances adjusted for missing data as calculated in PAUP

Item	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 CBS 130.79	–	0.064	0.140	0.139	0.142	0.138	0.195	0.191	0.181	0.207	0.207	0.212	0.209	0.264	0.255	0.230	0.262	0.265
2 263		–	0.146	0.149	0.148	0.140	0.195	0.211	0.205	0.218	0.218	0.223	0.224	0.286	0.267	0.241	0.279	0.271
3 AQ10			–	0.006	0.002	0.040	0.132	0.148	0.152	0.173	0.171	0.182	0.181	0.231	0.246	0.224	0.243	0.247
4 DSM 2222				–	0.008	0.047	0.139	0.150	0.155	0.182	0.180	0.191	0.190	0.235	0.249	0.231	0.241	0.245
5 ATCC 201056					–	0.043	0.134	0.150	0.154	0.175	0.173	0.184	0.183	0.233	0.248	0.226	0.245	0.249
6 CBS 131.31						–	0.142	0.157	0.159	0.178	0.181	0.190	0.186	0.240	0.260	0.241	0.250	0.252
7 DSM 2223							–	0.163	0.173	0.190	0.188	0.204	0.212	0.239	0.271	0.255	0.272	0.281
8 *L. microscopica*								–	0.105	0.158	0.154	0.171	0.184	0.202	0.230	0.204	0.256	0.261
9 *L. nodorum*									–	0.153	0.149	0.156	0.162	0.187	0.218	0.204	0.257	0.248
10 *A. quercinus* CBS 633.92										–	0.004	0.033	0.048	0.176	0.190	0.194	0.226	0.230
11 *A. humuli* ATCC 38616											–	0.031	0.046	0.170	0.186	0.189	0.226	0.223
12 *E. nigrum*												–	0.060	0.186	0.189	0.190	0.220	0.238
13 ATCC 201059													–	0.169	0.182	0.198	0.215	0.218
14 *A. alternata*														–	0.189	0.186	0.246	0.230
15 *L. maculans* Unity															–	0.122	0.214	0.238
16 *L. maculans* Leroy																–	0.207	0.237
17 *L. doliolum*																	–	0.254
18 *L. bicolor*																		–

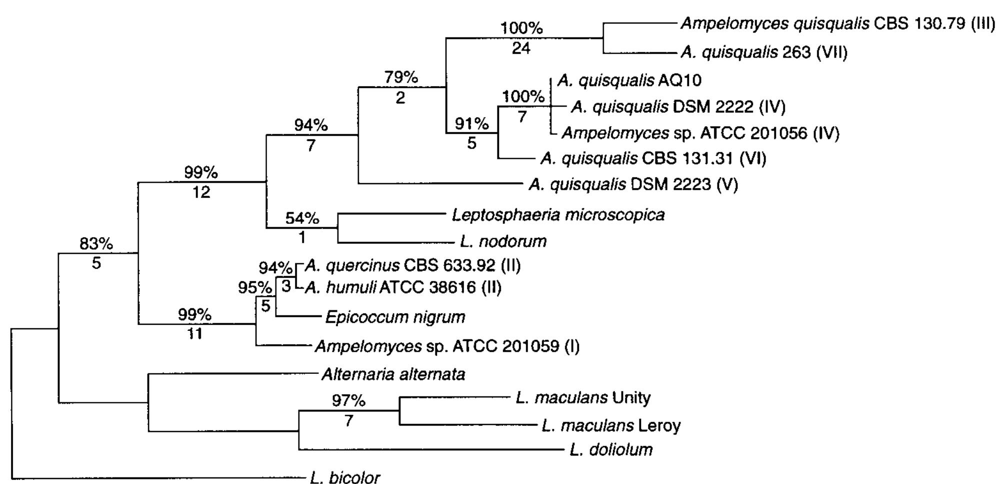

Fig. 1 A phylogram of one of the most-parsimonious trees based on sequences of the ITS region of the rDNA. Bootstrap confidence levels (%) are shown above, and decay indices below, the branches. *Roman numerals in parentheses* correspond to the RFLP group (Kiss 1997a)

trees recovered. In this tree the slow-growing *Ampelomyces* isolates form a clade that is distinct and well separated from the fast-growing *Ampelomyces* isolates and *E. nigrum.* These clades are well supported by bootstrap (94–99% confidence levels) and decay analyses. In addition, *L. nodorum, L. microscopica* and the slow-growing *Ampelomyces* isolates form another well-supported clade (99% confidence level). Trees produced from the weighting of transversions 1.1-, 2- and 3-times more than transitions were identical to the tree shown in Fig. 1. The 50% majority-rule consensus bootstrap tree differed from the tree depicted in Fig. 1 in a minor detail near the base of tree in which *A. alternata, L. doliorum,* and the clade containing *L. maculans* Unity and Leroy, formed an unresolved polychotomy with the clade containing the *Ampelomyces* isolates, *E. nigrum, L. microscopica* and *L. nodorum.*

Because of the uncertainty of alignment of the first 81 bp positions, these sites were excluded, and the data re-analyzed. In this partial data set, there were 129 potentially informative characters. The sequence-divergence values were slightly lower: among the slow-growing *Ampelomyces* isolates 0.2-16.3% (data not shown) and between the fast-growing isolates and *E. nigrum* 0.5–4.5%. Six most-parsimonious trees, each 468 steps long with a CI of 0.557 and RI of 0.685, were recovered. The strict consensus tree was similar to the trees recovered from the entire data set (see Fig. 1), except that the clade containing the *Ampelomyces* isolates, *E. nigrum, L. microscopica* and *L. nodorum* formed a trichotomy with *A. alternata* and the *L. doliorum* and *L. maculans* Unity and Leroy clade.

Other alignments were produced to accommodate different gap arrangements. In the subsequent analyses of complete and partial data sets, slightly different tree topologies with varied CI, RI, and bootstrap and decay values were obtained. However, the relationships of the slow-growing *Ampelomyces* isolates to each other and of the fast-growing *Ampelomyces* isolates to *E. nigrum* were unchanged and identical to that depicted in Fig. 1. Most of the differences in the trees occurred with the position of *A. alternata* and the collapse of the *L. microscopica* and *L. nodorum* clade.

Discussion

Both the high ITS sequence-divergence values (Table 3) and the phylogenetic relationships with other closely related taxa (Fig. 1) indicate that intracellular hyperparasites of powdery mildew fungi included in the genus *Ampelomyces* cannot, as is currently widely accepted, be treated as a single species. Intra- and inter-specific divergences in ITS sequences are variable in various fungal groups (e.g. O'Donnell 1992; Carbone and Kohn 1993; Seifert et al. 1995). However, the relatively high sequence divergence values (4.3–14.2%) among the *Ampelomyces* isolates belonging to the seven RFLP groups suggest that they each represent a different taxon. In contrast, isolates belonging to the same RFLP groups were characterized with low sequence-divergence values (< 1%) indicating that they are probably conspecific. Differences in morphological and cultural patterns (Kiss and Vajna 1995) and RFLP patterns (Kiss 1997a) also support the notion that some of the *Ampelomyces* isolates studied represent different taxa.

The slow-growing and fast-growing *Ampelomyces* isolates were separated into two, distinct and well-supported clades. Two types of growth patterns were observed during isolation of *Ampelomyces* hyperparasites under various cultural conditions (Mhaskar 1974; Mhaskar and Rao 1974; Rudakov 1979; Sz. Nagy and Vajna 1990; Kiss and Vajna 1995). Cladistic analyses indicate that the slow-growing and fast-growing isolates studied are not closely related and may not be congeneric. They may represent different lineages within the Leptosphaeriaceae that have both adapted to a hyperparasitic way of life in hyphae of the Erysiphaceae.

Our results show that the coelomycetous *Ampelomyces* spp., included in the Deuteromycetes because they lack a sexual stage, have their phylogenetic position in the Dothideales. Their ascomycetous nature was suggested earlier by electron microscopic studies of the septal pores (Hashioka and Nakai 1980; Kimbrough 1994). They are related primarily to *Leptosphaeria* spp. that represent two or three phylogenetic lineages within the Dothideales (Morales et al. 1993, 1995). The evolutionary position of *Ampelomyces* is not really surprising since the anamorphs of *Leptosphaeria* spp. belong to many coelomycetous genera; e.g. *Phoma,* and *Ampelomyces* hyperparasites produce *Phoma*-like pycnidia in senescent plant tissues (Yarwood 1939; Jarvis and Slingsby 1977; Kranz 1981) or in culture. The position of *Alternaria* could also be explained by the morphological patterns of its teleomorph (Jasalavich et al. 1995; Morales et al. 1995).

From ITS sequences available in databases, *E. nigrum* appears to be the closest relative to the three fast-growing *Ampelomyces* isolates studied. This relationship can not be easily explained by classical morphological observations. The only morphological data that could indicate a possible phylogenetic relationship between *E. nigrum* and pycnidial fungi is that the *Epicoccum* state of *Phoma epicoccina* is indistinguishable from *E. nigrum* (Booth 1980). Rollo et al. (1995) demonstrated a close phylogenetic relationship between *E. nigrum* and *L. maculans.*

Recently, Samson (1995) presented a long list of fungal taxa used in biological control having taxonomic problems of immediate practical importance. This study showed that pycnidial hyperparasites of powdery mildews known today as *A. quisqualis* should be added to that list. As a consequence of the 'single *Ampelomyces* species' concept, only one or a few *'A. quisqualis'* hyperparasites have been used in various biological control experiments instead of comparing the mycoparasitic activity of a large number of isolates. Phylogenetic differences identified in the present study suggest that differences could be found in the use of genetically diverse *Ampelomyces* isolates in biocontrol applications, a possibility that has not been previously exploited. In addition, an accurate molecular identification of different pycnidial hyperparasites of powdery mildews released in the field in various biological control experiments and commercial applications is clearly needed.

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