

Existence of different physiological forms within genetically diverse strains of *Ampelomyces quisqualis*

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Abstract Powdery mildew fungi are parasitized by strains of the genetically distinct *Ampelomyces quisqualis*. To investigate whether differences in the phylogeny and other cultural, morphological and physiological characteristics of these different strains are related to differences in their geographic origins or the host species from which they were isolated, several *A. quisqualis* strains isolated from different species of *Erysiphaceae* collected in different countries and possessing different ITS rDNA sequences were selected and characterized. The results revealed some significant variation among the selected strains, which provides evidence for the existence of different physiological forms within the *A. quisqualis* species. Two groups that display differential growth on artificial media were identified. These groups also differ in the morphology of their mycelium, but not in the morphology of their pycnidia and conidia. Temperature greatly affected the *in vitro* growth of the *A. quisqualis* strains and growth rate was closely correlated to colony color. Differences in the conidial germination of

distinct strains were observed during the recognition phase of the parasitic relationship. The germination of each of the investigated strains was greatly stimulated by all of the examined powdery mildew species and not only by the conidia of their original hosts. An Italian strain isolated from grapevine in the Trentino Alto-Adige region was identified as the strain that germinates the most quickly in the presence of powdery mildew conidia. Phylogenetic analysis revealed that these *A. quisqualis* strains can be classified into five different genetic groups, which generally correlate with the fungal host of origin and morphological and growth characteristics.

Keywords Adaptation · Biological control · Cultural and morphological characteristics · Fungal host · Genetic diversity · Mycoparasitic fungus

Introduction

Ampelomyces quisqualis is a widespread hyperparasite of powdery mildews (Falk *et al.* 1995a; Kiss 1998, 2003). The natural occurrence of *A. quisqualis* on various *Erysiphaceae* species has been reported in different geographic regions (Angeli *et al.* 2009b; Kiss 1997; Kiss *et al.* 2004; Rankovic 1997). Most research on *A. quisqualis* has focused on its potential use as a biocontrol agent against powdery mildews of various crops (Bélanger and Labbé 2002; Paulitz and Bélanger 2001; Sundheim and Tronsmo 1988; Szejnberg *et al.*

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1989). Its biology and biocontrol potential were reviewed by Kiss *et al.* (2004).

This mycoparasite invades and destroys host cytoplasm, killing the parasitized powdery mildew cells (Falk *et al.* 1995a, b; Hashioka and Nakai 1980; Kiss *et al.* 2004). Intracellular pycnidia of *A. quisqualis* are commonly found in hyphae, conidiophores and immature ascomata of powdery mildews (Kiss *et al.* 2004). These pycnidia vary in shape depending upon the fungal structure in which they are formed (Sundheim and Krekling 1982). Pycnidia contain cylindrical to spindle-shaped conidia, which are occasionally curved and two-spotted (Falk *et al.* 1995a). Recently, the microcyclic conidiogenesis of powdery mildews has been investigated (Kiss *et al.* 2009). When mildew colonies are treated with a suspension of *A. quisqualis* conidia, pycnidia are formed in microcyclic conidiophores, thereby accelerating the asexual reproduction of *A. quisqualis*.

The concentration of *A. quisqualis* conidia is an important factor affecting their germination. Germination has been shown to decrease dramatically when conidia are at a concentration of more than 10^6 conidia ml^{-1} , due to the production of self-inhibitory substances (Gu and Ko 1997). *A. quisqualis* conidia do not germinate well in sterile, distilled water. However, their germination is significantly enhanced in the presence of conidia of powdery mildew fungi (Sundheim 1982). The presence of host fungi is recognized by *A. quisqualis* and a water-soluble substance from conidia of powdery mildew fungi has been shown to stimulate the germination of *A. quisqualis* conidia *in vitro* (Gu and Ko 1997). After penetration, the hyphae of the mycoparasite continue to grow and produce their intracellular pycnidia after 5 to 8 days in the mycelia of their fungal host (Hashioka and Nakai 1980; Sundheim and Krekling 1982). High relative humidity and temperatures between 20° and 25°C enhance the growth and sporulation of *A. quisqualis*, but this mycoparasite survives and is active against powdery mildew even at temperatures below 12°C (Jarvis and Slingsby 1997; Philipp and Cruger 1979).

Ampelomyces quisqualis is known to be a slow-growing fungus with an *in vitro* radial growth rate of 0.5–1.0 mm d^{-1} on Czapek-Dox agar supplemented with 2% malt extract (MCzA) at 23°C (Kiss 1997; Kiss and Nakasone 1998). Until recently, *A. quisqualis* was often confused with several other species: *A.*

quercinus, *A. humuli*, *A. heraclii* and *Phoma glomerata*. Unlike *A. quisqualis*, these other powdery mildew mycoparasites do not produce intracellular pycnidia and they also grow more quickly, with *in vitro* radial growth rates of 3–4 mm d^{-1} (Kiss *et al.* 2004; Sullivan and White 2000). In the past, scientists identified all of these fast-growing strains as *A. quisqualis* (e.g. Kiss 1997; Mhaskar 1974). Later, a molecular phylogenetic study of rDNA ITS sequences (Kiss and Nakasone 1998) revealed that the slow-growing and fast-growing strains belong to two distinct groups. The slow-growing strains are genetically more diverse, whereas the fast-growing ones show a close phylogenetic relationship with *Epicoccum nigrum*, which is known to have a pycnidial, *Phoma*-like state (Kiss *et al.* 2004). It seems likely that the fast-growing strains are, in fact, *Phoma* species, whereas true *A. quisqualis* strains always grow slowly in culture and produce intracellular pycnidia in powdery mildew mycelium.

Several *A. quisqualis* strains are available from culture collections and one strain has been formulated, registered and commercialized under the trade name of AQ10 (Kiss 1997; Szejnberg 1993). Many molecular analyses based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (nrDNA) have revealed considerable genetic diversity among *A. quisqualis* strains (Angeli *et al.* 2009a; Liang *et al.* 2007; Park *et al.* 2010; Szentiványi *et al.* 2005). Recently, ITS sequences and microsatellite markers have been used to show that a set of *A. quisqualis* populations found in apple powdery mildew (*Podosphaera leucotricha*) are quite genetically distinct from populations collected from several other powdery mildew species infecting other plant species (Kiss *et al.* 2011). Although the genetic diversity within the *A. quisqualis* species has already been characterized, we still do not know whether the differences in the phenotypic characteristics of different strains of *A. quisqualis* are related to their phylogeny.

The morphological and cultural characteristics of this hyperparasite on different fungal hosts and plants have been reported to be extremely variable (Belsare *et al.* 1980; Shin and Kyeung 1994; Speer 1978). Some older studies focused on the shape and size of the pycnidia and conidia on culture media (e.g. Mhaskar 1974; Mhaskar and Rao 1974) and fungal hosts (e.g. Kiss 1997; Rankovic 1997). However, all of these studies were carried out when fast-growing

strains (*Phoma glomerata* and *Ampelomyces* spp.) were still classified as *A. quisqualis*. Therefore, a thorough investigation of *A. quisqualis sensu strictu* is lacking. More physiological and phenotypic information is required to complete the picture of the genetic differences that have been identified in previous studies (Kiss *et al.* 2011; Liang *et al.* 2007; Park *et al.* 2010).

In the present work, we selected several *A. quisqualis* strains from different hosts and geographic regions, all characterized by the formation of intracellular pycnidia and slow radial growth at room temperature *in vitro* (defined as Type II; Kiss 1997), features corresponding to the characterization of *A. quisqualis sensu strictu*. We investigated the cultural, morphological and growth characteristics and phylogenetic relations (ITS) of these strains. The objectives of this study were to determine whether the host or site of origin of the strains or their cultural, morphological and/or growth characteristics are related to their phylogenetic group, which would indicate an adaptation to the host or geographic area. Furthermore, some physiological tests were performed as we looked for evidence of any host specificity or temperature adaptation of strains affecting the recognition phase of the host–parasite interaction.

Materials and methods

A total of 28 *A. quisqualis* strains isolated from different crops, sites and powdery mildew species were included in this study (Table 1). We selected strains isolated from grapevine (*Vitis vinifera*), cucumber (*Cucumis sativus*) and apple (*Malus domestica*) in different geographic regions, as well as strains isolated from wild and/or ornamental species. Only slow-growing strains, e.g. Type II (0.5–1.0 mm d⁻¹) (Kiss 1997), producing intracellular pycnidia within powdery mildew mycelia were selected. The strains were obtained from culture collections (ATCC, American Type Culture Collection, Rockville, MD, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, the Netherlands; CABI, Commonwealth Agricultural Bureaux International, Egham, UK; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) or provided by individual scientists (L. Kiss, PPI, Plant Protection Institute of the Hungarian Academy of Sciences, Budapest,

Hungary; D. Angeli, FEM, Fondazione Edmund Mach, S. Michele all'Adige, Italy). For phylogenetic comparison, 15 sequences of ITS rDNA of strains of *A. quisqualis* were retrieved from GenBank (Table 2). Strains were selected according to the fungal host of origin. The selected strains represent the four major clades reported in a recent study by Park *et al.* (2010).

Cultural, morphological and physiological characteristics The cultural behavior and morphological characteristics of the pycnidia and conidia of the 28 selected *A. quisqualis* strains were evaluated on six different types of media: potato dextrose agar [PDA] (Oxoid, Hampshire, UK, 39 g l⁻¹ twice-distilled water), sucrose nutrient agar (50 g sucrose, 28 g nutrient agar, twice-distilled water to 1l), yeast malt dextrose agar (10 g dextrose, 5 g peptone, 3 g yeast extract, 3 g malt extract, 20 g agar, twice-distilled water to 1l), nutrient agar (Oxoid, 28 g l⁻¹ twice-distilled water), Czapek agar (Oxoid, 33.4 g l⁻¹ twice-distilled water) and cornmeal agar (Oxoid, 17 g l⁻¹ twice-distilled water)

The petri dish cultures of fungus were prepared with 100 µl of a suspension of sterile distilled water containing 10² conidia ml⁻¹. The color of the mycelia and pycnidia was assessed by visual observation of colonies grown for 14 days at 25°C in the dark. We measured the length and width of 60 pycnidia and 60 conidia per strain (20 per replicate) on glass slides under a light microscope (Hund Wetzlar H 600LL, Wetzlar, Germany). The radial growth rate of the *A. quisqualis* strains was evaluated at temperatures of 10°, 15°, 20°, 25° and 30°C on petri dishes containing PDA that were inoculated as described above. Cultures were incubated in the dark for 30 days and the radial growth of the colonies (20 per replicate) was evaluated by measuring the diameter of each colony twice a week. All of the experiments were conducted with three replicates (plates) per strain.

The recognition of the fungal host by *A. quisqualis* strains at different temperatures was studied. The effects of the conidia of four powdery mildew species, *Podosphaera xanthii* (from cucumber), *Erysiphe necator* (from grapevine), *Podosphaera aphanis* (from strawberry) and *Podosphaera leucotricha* (from apple), on the germination rates of five selected *A. quisqualis* strains were evaluated. Each of the tested strains belongs to a different genetic (ITS) group (identified in this study) and was collected from a

Table 1 Designation, plant host, area and year of isolation, source and GenBank accession numbers of *Ampelomyces quisqualis* strains included in this study. All strains are TypeII (growth rate: 0.5–1.0 mm d⁻¹) and were acquired from culture collections (ATCC, CBS, CABI, DSMZ) or provided by individual scientists (L. Kiss, PPI; D. Angeli, FEM)

Designation	Host fungus	Host plant	Geographical origin and year of isolation	Source ^z	GenBank accession no. ^y
ATCC200245	<i>Erysiphe necator</i>	<i>Vitis vinifera</i>	New York, USA, 1989	ATCC	AF126817
ATCC200246	<i>E. necator</i>	<i>V. vinifera</i>	New York, USA, 1991	ATCC	HQ108030
ATCC200247	<i>E. necator</i>	<i>V. vinifera</i>	New York, USA, 1991	ATCC	HQ108031
ATCC200248	<i>E. necator</i>	<i>V. vinifera</i>	New York, USA, 1991	ATCC	HQ108032
ATCC200249	<i>E. necator</i>	<i>V. vinifera</i>	New York, USA, 1991	ATCC	HQ108033
ATCC200250	<i>E. necator</i>	<i>V. vinifera</i>	New York, USA, 1991	ATCC	HQ108034
ITA1	<i>E. necator</i>	<i>V. vinifera</i>	Italy, 2007	FEM	HQ108047
ITA2	<i>E. necator</i>	<i>V. vinifera</i>	Italy, 2007	FEM	HQ108048
ITA3	<i>E. necator</i>	<i>V. vinifera</i>	Italy, 2007	FEM	HQ108049
ITA4	<i>E. necator</i>	<i>V. vinifera</i>	Italy, 2007	FEM	HQ108050
ITA5	<i>E. necator</i>	<i>V. vinifera</i>	Italy, 2007	FEM	HQ108051
CBS128.79	<i>Erysiphe cichoracearum</i>	<i>Cucumis sativus</i>	Canada, 1975	CBS	HQ108037
CBS129.79	<i>E. cichoracearum</i>	<i>C. sativus</i>	Canada, 1975	CBS	HQ108038
CBS130.79	<i>E. cichoracearum</i>	<i>C. sativus</i>	Canada, 1975	CBS	HQ108039
CBS131.79	<i>E. cichoracearum</i>	<i>C. sativus</i>	Canada, 1975	CBS	HQ108040
DSM2222	<i>E. cichoracearum</i>	<i>C. sativus</i>	Germany, unknown ^w	DSMZ	U82450
MYA-3389	<i>Podospaera leucotricha</i>	<i>Malus domestica</i>	Hungary, 1995	PPI	AY663815
MYA-3391	<i>P. leucotricha</i>	<i>M. domestica</i>	Hungary, 1995	PPI	HQ108043
MYA-3394	<i>P. leucotricha</i>	<i>M. domestica</i>	United Kingdom, 2002	PPI	HQ108044
MYA-3395	<i>P. leucotricha</i>	<i>M. domestica</i>	Germany, 2002	PPI	AY663817
MYA-3398	<i>P. leucotricha</i>	<i>M. domestica</i>	United Kingdom, 2002	PPI	HQ108045
AQ10	Powdery mildew ^x	<i>Chata edulis</i>	Israel, unknown ^w	Ecogen Italia	AF035783
DSM2225	<i>Erysiphe heraclii</i>	<i>Daucus</i> sp.	Germany, unknown ^w	DSMZ	HQ108042
DSM4624	<i>Sphaerotheca fuliginea</i>	<i>Leontodon</i> sp.	Germany, unknown ^w	DSMZ	HQ108041
CABI272851	Powdery mildew ^x	<i>Schinus molle</i>	Ecuador, 1983	CABI	HQ108036
CABI380234	Powdery mildew ^x	<i>Alliaria petiolata</i>	Switzerland, 1999	CABI	HQ108035
ATCC201056	<i>Oidium hortenisiae</i>	<i>Lycium halimifolium</i>	Hungary, 1990	ATCC	AF035781
MYA-3401	<i>Oidium hortenisiae</i>	<i>Hydrangea macrophylla</i>	United Kingdom, 1999	PPI	HQ108046

^z ATCC, American Type Culture Collection, Rockville, MD, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, the Netherlands; CABI, Commonwealth Agricultural Bureaux International, Egham, UK; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; FEM, Fondazione Edmund Mach, S. Michele all'Adige, Italy; PPI, Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary

^y GenBank accession numbers of the rDNA ITS sequences

^x Unknown species

^w Data not available

different host plant in a different geographic area (AQ10, ITA3, CBS129.79, MYA-3391 and MYA-3401). Each strain was paired with an isolate of the fungal host from which it was originally isolated and three other powdery mildews. Sterile distilled water suspensions of the conidia of each powdery mildew

strain (1×10^5 conidia ml⁻¹) together with each *A. quisqualis* strain (1×10^5 conidia ml⁻¹) were prepared, for a total of 20 combinations. Suspensions of only *A. quisqualis* conidia in sterile distilled water were used as controls. Six drops (10 µl each) per combination (three replicates) were put onto a glass slide, which

Table 2 GenBank accession numbers of the rDNA ITS sequences, plant hosts, area and year of isolation and source of published *Ampelomyces quisqualis* sequences included in the ITS sequence analysis

GenBank accession no. ^z	Host fungus	Host plant	Geographical origin and year of isolation	Source
GQ324149 ^y	<i>Erysiphe necator</i>	<i>Vitis vinifera</i>	Seoul, South Korea, 2007	Park <i>et al.</i> (2010)
GQ324144 ^y	<i>E. necator</i>	<i>Vitis flexuosa</i>	Jeju, South Korea, 2006	Park <i>et al.</i> (2010)
DQ490750	<i>Podosphaera xanthii</i>	<i>Cucurbita pepo</i>	China, 2002	Liang <i>et al.</i> (2007)
DQ490752	<i>P. xanthii</i>	<i>Cucumis moschata</i>	China, 2002	Liang <i>et al.</i> (2007)
DQ490755	<i>P. xanthii</i>	<i>Cucumis sativus</i>	China, 2002	Liang <i>et al.</i> (2007)
DQ490759	<i>P. xanthii</i>	<i>Cucurbita maxima</i>	China, 2002	Liang <i>et al.</i> (2007)
AY663816	<i>Podosphaera leucotricha</i>	<i>Malus domestica</i>	Hungary, 2000	Szentiványi <i>et al.</i> (2005)
AY663818	<i>P. leucotricha</i>	<i>M. domestica</i>	UK, 2002	Szentiványi <i>et al.</i> (2005)
AY663819	<i>P. leucotricha</i>	<i>M. domestica</i>	UK, 2002	Szentiványi <i>et al.</i> (2005)
AY663820	<i>P. leucotricha</i>	<i>M. domestica</i>	Germany, 2002	Szentiványi <i>et al.</i> (2005)
AY663821	<i>P. leucotricha</i>	<i>M. domestica</i>	Hungary, 2002	Szentiványi <i>et al.</i> (2005)
GQ324095 ^y	<i>Erysiphe ranunculi</i>	<i>Clematis apiifolia</i>	Seogwipo, South Korea, 2006	Park <i>et al.</i> (2010)
GU329997 ^y	<i>Golovinomyces cichoracearum</i>	<i>Zinnia elegans</i>	Yangpyeong, South Korea, 2008	Park <i>et al.</i> (2010)
DQ490762	<i>Arthrocladiella mougeotii</i>	<i>Lycium chinense</i>	China, 2003	Liang <i>et al.</i> (2007)
DQ490767	<i>Oidium</i> sp.	<i>Castanopsis</i> sp.	China, 2002	Liang <i>et al.</i> (2007)

^z GenBank accession numbers of the rDNA ITS sequences

^y Strains identified in the maximum-likelihood tree with the same isolate numbers used by Park *et al.* (2010)

was placed in a petri dish (r.h. = 100%) and stored in a dark incubator kept at 25°C, which is the temperature that Gu and Ko (1997) used in their study of the factors affecting the germination of *A. quisqualis* conidia. The germination rates and germ-tube elongation of 150 conidia per strain (three replicates, 50 conidia per replicate) were evaluated under a light microscope after 24 h.

The effect of temperature on the germination of *A. quisqualis* conidia in the presence of the fungal host was evaluated, by testing two powdery mildew species (*P. xanthii* from cucumber and *E. necator* from grapevine) and the two *A. quisqualis* strains that were the most and least responsive to powdery mildew conidia in the previous experiment. Both the germination rate and the tube elongation of those strains were recorded at three temperatures (15°, 20° and 25°C) using the method described above.

Phylogenetic analysis The phylogenetic relations of the 28 strains analyzed in the present study and another 15 strains selected for phylogenetic analysis were inferred from maximum-likelihood (ML) and maximum-parsimony (MP) analyses in which the

sequences of their respective ITS rDNA regions were compared (White *et al.* 1990). The additional 15 sequences of ITS rDNA from *A. quisqualis* strains that were analyzed by Park *et al.* (2010) were retrieved from GenBank (Table 2). The strains isolated by Park *et al.* (2010), whose GenBank accession numbers begin with GQ and GU, were identified here with the same isolate numbers used by Park *et al.* (2010), to make it easier to recognize them across the two studies (SMKC22963 = GQ324149, SMKC22519 = GQ324144, SMKC22511 = GQ324095, SMKC23914 = GU329997).

The 28 selected strains (Table 1) were grown on PDA for 21 days at 25°C. DNA was extracted from approximately 200 µg of homogenized, lyophilized mycelium, using the Nucleo Spin Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The ITS region of the nuclear ribosomal DNA was amplified using ITS1 and ITS4 fungal-specific primers (Gardes and Bruns 1993). PCR was performed using the Gene Amp PCR System 9700 (PerkinElmer, Waltham, MA, USA). PCR products were purified using ExoSAP-IT enzymes (USB Corporation, Staufen, Germany)

and sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Both DNA strands were sequenced with the primers that had been used for PCR amplification and electrophoresis was carried out using an ABI 3130xl Genetic Analyzer (PerkinElmer). The consensus sequence was assembled using Pregap4 and Gap4 (Staden *et al.* 2000; <http://staden.sourceforge.net/>). Blast searches were performed in the NCBI/GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/>) us-

ing the program Megablast to find long stretches of alignment between very similar nucleotide sequences. The ClustalW2 program (Larkin *et al.* 2007; <http://www.ebi.ac.uk/Tools/clustalw2/index.html>) was used to construct multiple sequence alignments and to identify evolutionary relationships among the input sequences. ML and MP analyses based on ITS region sequences were conducted using the Mega4 program (Tamura *et al.* 2007; <http://www.megasoftware.net/mega.html>) and a p-distance model (Nei and Kumar

Table 3 Morphological characteristics of the *Ampelomyces quisqualis* strains included in this study

Designation	Colony color ^z		Length and width ($\mu\text{m} \pm \text{SD}$) ^y	
	Mycelia	Pycnidia	pycnidium	conidium
ATCC200245	Olive green	Light/dark olive green	65±25×38±15	8.9±1.9×2.7±0.4
ATCC200246	Olive green	Light/dark olive green	58±18×31±12	8.4±1.6×2.9±0.4
ATCC200247	Olive green	Light/dark olive green	57±15×32±14	7.9±1.3×2.8±0.4
ATCC200248	Olive green	Light/dark olive green	58±18×31±12	8.5±1.9×2.8±0.4
ATCC200249	Olive green	Light/dark olive green	64±23×40±11	8.4±1.7×2.9±0.5
ATCC200250	Olive green	Light/dark olive green	59±11×32±8	8.5±1.8×2.8±0.4
ITA1	Dark brown	Light/dark brown	72±23×42±13	8.4±1.6×2.8±0.4
ITA2	Dark brown	Light/dark brown	67±9×39±12	9.8±1.8×2.9±0.5
ITA3	Olive green	Light/dark olive green	54±15×34±11	8.0±1.7×2.9±0.4
ITA4	Olive green	Light/dark olive green	56±16×33±7	7.3±1.5×2.9±0.5
ITA5	Olive green	Light/dark olive green	64±13×37±9	7.3±1.5×2.7±0.4
CBS128.79	Dark brown	Light/dark brown	59±10×35±10	7.1±1.3×2.6±0.2
CBS129.79	Dark brown	Light/dark brown	66±14×41±7	7.3±1.5×2.8±0.5
CBS130.79	Dark brown	Light/dark brown	64±9×38±9	9.0±1.6×2.9±0.5
CBS131.79	Dark brown	Light/dark brown	71±18×37±8	9.1±1.9×2.9±0.4
DSM2222	Dark brown	Light/dark brown	67±16×41±9	7.9±1.2×2.7±0.4
MYA-3389	Olive green	Light/dark olive green	72±20×38±12	7.9±1.0×2.6±0.2
MYA-3391	Olive green	Light/dark olive green	61±16×34±9	7.8±1.8×2.7±0.4
MYA-3394	Olive green	Light/dark olive green	69±15×36±11	8.7±1.8×2.8±0.4
MYA-3398	Olive green	Light/dark olive green	65±10×34±7	9.1±1.8×2.8±0.4
MYA-3395	Olive green	Light/dark olive green	66±17×37±10	9.6±1.6×2.7±0.4
AQ10	Dark brown	Light/dark brown	55±10×32±5	7.5±1.6×2.6±0.3
DSM2225	Dark brown	Light/dark brown	62±15×36±10	9.6±1.4×2.9±0.4
DSM4624	Dark brown	Light/dark brown	58±9×36±7	7.9±1.6×2.8±0.4
CABI272851	Dark brown	Light/dark brown	66±15×35±11	8.5±1.6×2.7±0.4
CABI380234	Olive green	Light/dark olive green	56±18×34±12	9.5±1.8×3.0±0.4
ATCC201056	Olive green	Light/dark olive green	58±17×37±12	8.1±1.2×2.6±0.2
MYA-3401	Dark brown	Light/dark brown	73±19×44±14	10.6±2.6×2.9±0.5

^z Colors of mycelia and pycnidia on the six tested agar media. Three agar plates per strain were examined visually following 14 days of incubation at 25°C in the dark

^y Values are means from cultures grown on the six media (360 pycnidia and 360 conidia per strain). No significant differences between media were detected when Tukey's test was applied ($P \geq 0.05$). Standard deviations (SD) of the means are reported

2000) was used to calculate within-group and between-group distances. A phylogenetic tree inferred from the ITS rDNA region sequences was constructed using the TreeView program, version 1.6.6 (Page 1996; <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). *P. glomerata* (AF126816), *A. quercinus* (AF035778) and *A. humuli* (AF035779) were also included in the model.

Clustering and statistical analysis Morphological experiments (size of pycnidia and conidia, Table 3) were performed in three replicates per strain and medium and standard deviations of the averages were calculated. A one-way analysis of variance (ANOVA) was performed on log-transformed and arcsin-transformed data. If significant differences were detected, treatment means (strains and media) were separated using Tukey's test ($\alpha=0.05$).

Growth tests involved two independent experiments with three replicates per experiment. A two-way ANOVA was performed on log-transformed and arcsin-transformed data from two experiments and revealed no significant Experiment \times Treatment (temperature) interaction. Therefore, cluster analyses were performed using a software package for multivariate data analysis capable of performing K-means cluster analyses (Multi-Experiment Viewer, TMEV). The Euclidean distance between each of the samples within a given cluster and their respective cluster centroid was calculated. The angular coefficients (a) and the correlation coefficients (r) of the linear regression curves were also calculated. Data from the two experiments were pooled and averages of six replicates are presented in Table 4 and Fig. 1. Statistical analyses were performed using Statistica software 7.0 (Statsoft, Tulsa, OK, USA). Significant differences between treatments (strains and temperatures) were separated using Scheffé's test ($\alpha=0.05$).

Germination tests involved two independent experiments with three replicates per experiment and 50 conidia per replicate. Two-way ANOVA was performed on both the log-transformed and arcsin-transformed data from the two experiments and revealed no significant Experiment \times Treatment (temperature) interaction. Therefore, data from the two experiments were pooled. Averages of six replicates are presented in Figs. 2 and 3. Significant differences between treatments (strains and powdery mildews) were separated using Tukey's test ($\alpha=0.05$).

Table 4 Growth rates of the *Ampelomyces quisqualis* strains included in this study at five different temperatures. Data are expressed in terms of the angular coefficients (mm d^{-1}) of the linear regression curves

ID code	Temperature ($^{\circ}\text{C}$)				
	10	15	20	25	30
AQ10	0.3246 ^a	0.8187	1.0257	0.8152	<i>0.1384</i>
ITA1	0.3699	0.7141	0.8294	0.7525	<i>0.0875</i>
ITA2	0.3578	0.7813	0.8526	0.6817	<i>0.2126</i>
DSM2222	0.3686	0.6964	0.8047	0.6698	<i>0.1545</i>
DSM2225	0.3263	0.8272	0.8024	0.7044	<i>0.0799</i>
DSM4624	0.4339	0.7454	0.7851	0.6732	0.2862
CBS128.79	0.4276	0.7720	0.8145	0.7617	0.3303
CBS129.79	0.4089	0.7638	0.7545	0.7360	0.3185
CBS130.79	0.3701	0.7816	0.8032	0.6985	0.3271
CBS131.79	0.3543	0.8004	0.8473	0.7136	0.3646
CABI272851	0.3224	0.7148	0.8130	0.7700	0.2798
ITA3	0.3797	<i>0.5044</i>	<i>0.4922</i>	<i>0.5287</i>	0.3010
ITA4	<i>0.2459</i>	<i>0.4308</i>	<i>0.4394</i>	<i>0.6286</i>	0.2697
ITA5	<i>0.2637</i>	<i>0.4872</i>	<i>0.4517</i>	<i>0.5222</i>	0.2492
ATCC200246	<i>0.2343</i>	<i>0.5359</i>	<i>0.5398</i>	<i>0.4925</i>	0.3099
ATCC200245	<i>0.2567</i>	<i>0.3929</i>	<i>0.5671</i>	<i>0.5349</i>	<i>0.1711</i>
ATCC200247	<i>0.2809</i>	<i>0.4464</i>	<i>0.5935</i>	<i>0.5977</i>	<i>0.0825</i>
ATCC200248	0.3292	<i>0.4676</i>	<i>0.5405</i>	<i>0.5447</i>	<i>0.2176</i>
ATCC200249	<i>0.1957</i>	<i>0.4747</i>	<i>0.4604</i>	<i>0.4932</i>	<i>0.1180</i>
ATCC200250	<i>0.1805</i>	<i>0.3430</i>	<i>0.4474</i>	<i>0.4816</i>	<i>0.0692</i>
MYA-3389	<i>0.2448</i>	<i>0.4805</i>	<i>0.5008</i>	<i>0.5270</i>	<i>0.1371</i>
MYA-3391	<i>0.2856</i>	<i>0.5062</i>	<i>0.5589</i>	<i>0.5738</i>	<i>0.1389</i>
MYA-3394	0.3191	<i>0.4697</i>	<i>0.4402</i>	<i>0.5269</i>	<i>0.1736</i>
MYA-3395	<i>0.2061</i>	<i>0.5545</i>	<i>0.6459</i>	<i>0.5956</i>	<i>0.1616</i>
MYA-3398	<i>0.2763</i>	<i>0.5510</i>	<i>0.6349</i>	<i>0.6290</i>	<i>0.1717</i>
CABI380234	<i>0.1831</i>	<i>0.4107</i>	<i>0.5517</i>	<i>0.6213</i>	<i>0.1074</i>
ATCC201056	0.3412	<i>0.5259</i>	<i>0.5712</i>	<i>0.5294</i>	<i>0.1092</i>
MYA-3401	<i>0.2384</i>	<i>0.4486</i>	<i>0.5264</i>	<i>0.4797</i>	<i>0.1249</i>

^a Strains belonging to the slow-growing and very slow-growing clusters are listed in bold and italics, respectively. Radial growth was measured twice a week for 30 d. K-means clustering using the software TMEV (Multi-Experiment Viewer) was based on the Euclidean distance measured between each of the strains at each temperature. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment

Results

Cultural, morphological and physiological characteristics Cultural and morphological analyses of *A. quisqualis* strains indicated that two different

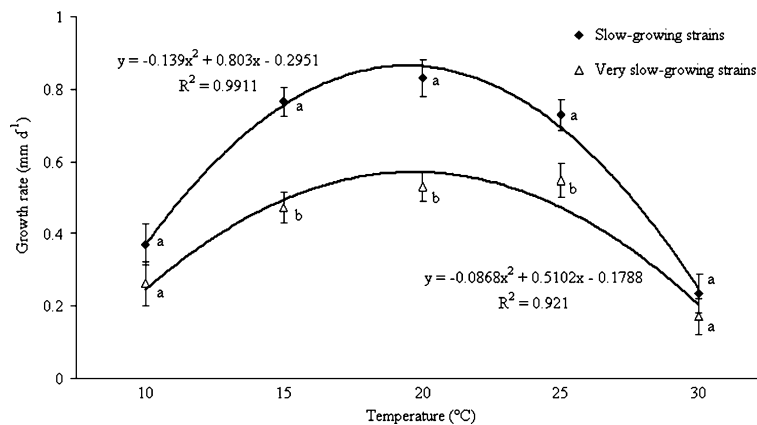


Fig. 1 Effect of temperature (10°, 15°, 20°, 25° and 30°C) on the growth of *Ampelomyces quisqualis* strains over 30 days on potato dextrose agar. The radial growth rates of 28 strains, which divided into slow-growing and very slow-growing groups at each temperature, are presented (pooled data). K-means clustering using the software TMEV (Multi-Experiment

Viewer) was based on the Euclidean distance between each of the strains at each temperature. Significant differences between groups were separated by ANOVA. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment. Bars represent the standard deviation of the six replicates

colony types can be distinguished after 14 d on all tested media: *A. quisqualis* strains that form dark-brown colonies and those that form olive-green colonies (mycelium and pycnidia; Table 3). Furthermore, mycelial growth and pycnidial and conidial formation were all greatly increased on three of the tested media (PDA, sucrose nutrient agar and yeast malt dextrose agar), in comparison with the other media. The study of morphology of pycnidia and conidia (Table 3) revealed differentiation in color and dimensions of the fruiting bodies of the tested strains. In all of the examined strains, the color and shape of the pycnidia varied from light to dark (brown or green) and from ovoid to ellipsoid, with increasing age of the culture. The length and width of the pycnidia ranged from 30 to 95 μm and from 25 to 55 μm , respectively. A great deal of variation in width and length was observed among the pycnidia of individual strains, but no significant differences were found between strains ($P=0.112$). The conidia of the different strains also varied in size. They ranged from 5.5 to 14.5 μm in length and from 2.3 to 3.5 μm in width. However, there were no significant differences between strains according to Tukey's test ($P=0.073$).

Temperature greatly affected the growth rate of *A. quisqualis* growth on PDA. The highest radial growth rates for all strains were measured between 15° and 25°C. At the extreme temperatures of 10° and 30°C, sharp reductions were observed in the growth rates (Fig. 1). K-means clustering based on Euclidean

distances showed that at temperatures of 15°, 20° and 25°C, the slow-growing Type II strains (Kiss 1997) could be further clustered into two different growth-rate groups that were significantly different from each other. At all of these temperatures, the two groups included the same isolates, which we refer to as slow-growing (0.70–0.82 mm d^{-1}) and very slow-growing (0.34–0.63 mm d^{-1}) (Table 4). At 10° and 30°C, K-means clustering analysis revealed two growth-rate classes, which do not include exactly the same strains as the groups observed at the other tested temperatures (Table 4). However, no statistically significant differences were observed between the two classes ($P=0.09$). At 10°C, the strains grew at a rate of 0.18–0.43 mm d^{-1} , whereas at 30°C they grew at a rate of 0.07–0.33 mm d^{-1} .

Our results indicate that, for all of the tested strains except MYA-3401, there is a relationship between the growth rates at 15°, 20° and 25°C and colony color. Strains that grew fairly slowly at these temperatures formed dark-brown colonies and strains that grew very slowly at these temperatures formed olive-green colonies. However, even with the limited number of strains examined, it is possible to demonstrate a relationship between growth rate and the original fungal host. Strains isolated from apple, grapevine and cucumber plants belonged to the same growth rate group at temperatures between 15° and 25°C. On the other hand, no relationship between the growth rate and the geographic origin of the strains was observed.

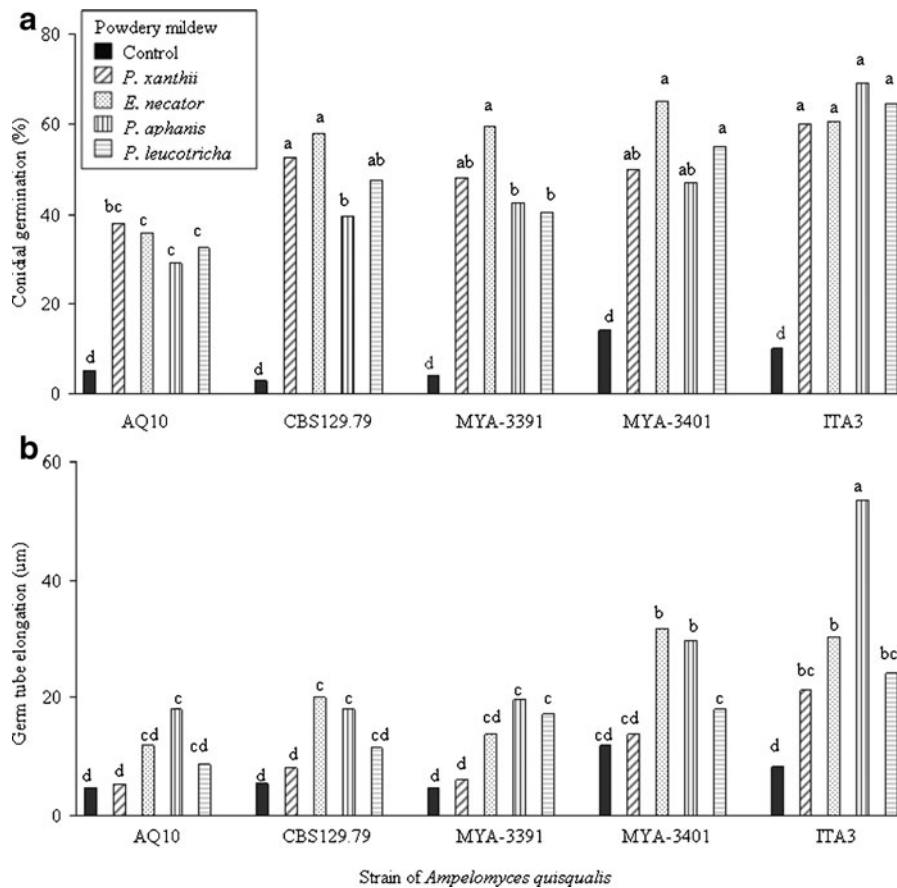


Fig. 2 Effects of different powdery mildew species (*Podosphaera xanthii* from cucumber, *Erysiphe necator* from grapevine, *Podosphaera aphanis* from strawberry and *Podosphaera leucotricha* from apple) on the (a) conidial germination (%) and (b) tube elongation (μm) of five selected strains of *Ampelomyces quisqualis*. Measurements were taken in aqueous suspensions containing mixtures of *A. quisqualis* and powdery mildew conidia that had been incubated together for 24 h at 25°C.

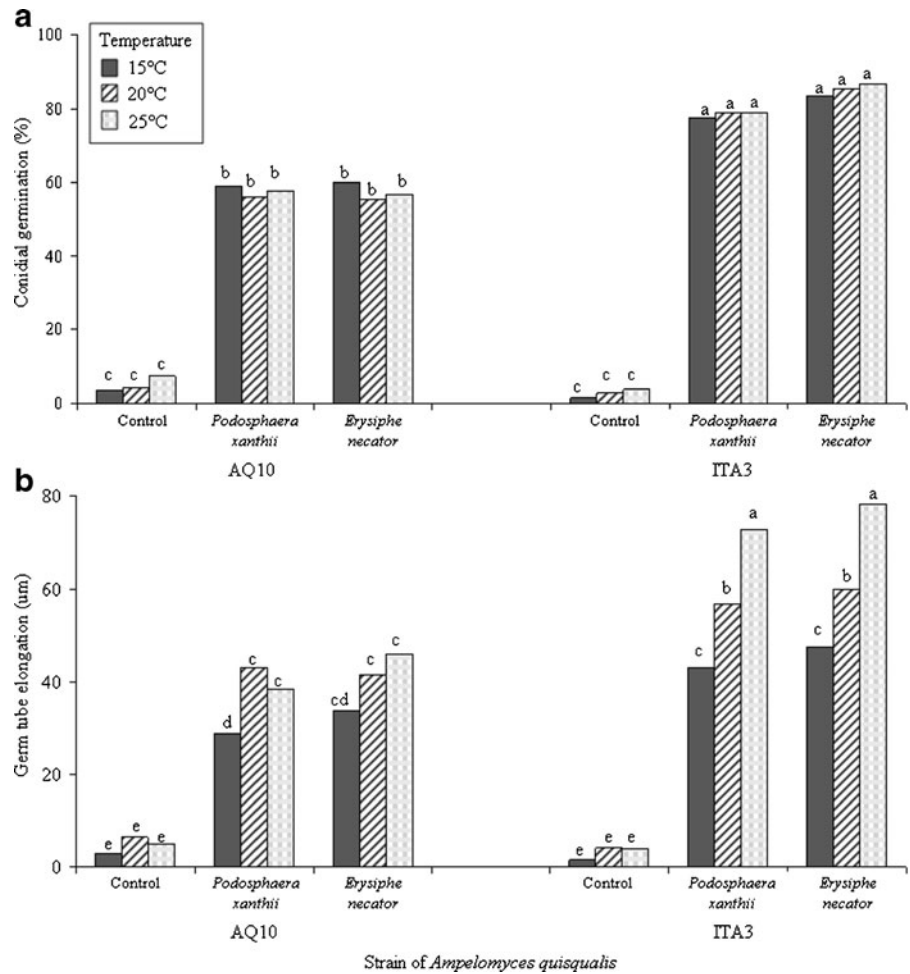
Physiological tests with different powdery mildew species and *A. quisqualis* strains showed that the germination rate and germ-tube length of *A. quisqualis* conidia increased greatly in the presence of powdery mildew conidia. This stimulation of germination is not powdery mildew species-specific. All of the tested powdery mildews increased the germination rate and tube elongation of the *A. quisqualis* strains isolated from different host species (Fig. 2a, b). However, in some cases, significant differences in the induction of germination were detected among the different powdery mildews. For example, the conidial germination of CBS129.79 (originally isolated from cucumber) was stimulated more strongly by grapevine powdery mildew

and cucumber powdery mildew than by strawberry powdery mildew. The conidial germination of MYA-3391 (originally isolated from apple) was most strongly induced by grapevine powdery mildew and was less affected by strawberry and apple powdery mildews. We observed big differences among the conidial germination rates of the different strains, independent of the powdery mildew species. The conidial germination of ITA3 and MYA-3401 was strongly induced by conidia of all of the tested powdery mildew species (both in terms of germination rate and tube elongation), whereas AQ10 was generally the least responsive.

Identical column designs indicate exposure to the same powdery mildew species. Within strains, columns labeled with a common letter (a-d) do not differ significantly ($P \leq 0.05$) according to Tukey's test. Three replicates (50 conidia per replicate) of each strain were evaluated. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment

In the second experiment, investigating the effect of temperature, it was again observed that, in the

Fig. 3 Effect of temperature on the (a) conidial germination (%) and (b) tube elongation (μm) of two selected strains of *Ampelomyces quisqualis* in the presence of two different powdery mildew species (*Podosphaera xanthii* from cucumber and *Erysiphe necator* from grapevine). Measurements were taken in aqueous suspensions containing mixtures of *A. quisqualis* and powdery mildew conidia that had been incubated for 24 h at three different temperatures (15°, 20° and 25°C). Identical column designs indicate incubation at the same temperature. Within strains, columns labeled with the same letter (a–e) do not differ significantly ($P \leq 0.05$) according to Tukey's test. Three replicates (50 conidia per replicate) of each strain were evaluated. Presented values are means of six replicates derived from two independent experiments with three replicates per experiment



absence of powdery mildew conidia, the germination rates and germ-tube elongation of strains ITA3 and AQ10 were very poor (Fig. 3a, b). Both parameters were strongly enhanced in the presence of powdery mildew conidia, with no statistical differences between the effects of *E. necator* and those of *P. xanthii*. As in the previous experiment, ITA3 was significantly more induced by powdery mildew conidia than AQ10 (Fig. 3a, b). We did not observe any changes in the general behavior of the two *A. quisqualis* strains at the different tested temperatures. In fact, temperature (within the tested range) had no effect on germination rate, but did seem to affect tube length. In the presence of powdery mildew conidia, tube elongation for both strains was always greater at the two higher temperatures (20° and 25°C) than at 15°C.

In these two experiments, AQ10 exhibited significantly lower germination rates and less tube elongation

in the presence of powdery mildew conidia, as compared to the strain ITA3 (Fig. 3a, b). However, on agar plates incubated at the examined temperatures, AQ10 and ITA3 were classified as slow-growing and very slow-growing, respectively. Thus, the growth rates of strains of *A. quisqualis* on artificial media are not correlated with their germination rates and tube elongation in the presence of a host fungus.

Phylogenetic analysis In the phylogenetic analysis, the 43 *A. quisqualis* strains clustered into five different groups (1–5), revealing genetic diversity among the *A. quisqualis* strains included in the present work (Fig. 4). Those strains with similar ITS sequences were often isolated from the same powdery mildew host. In fact, strains isolated from powdery mildew of apple (clade 3), powdery mildew of cucumber (clade 1) and powdery mildew of grapevine

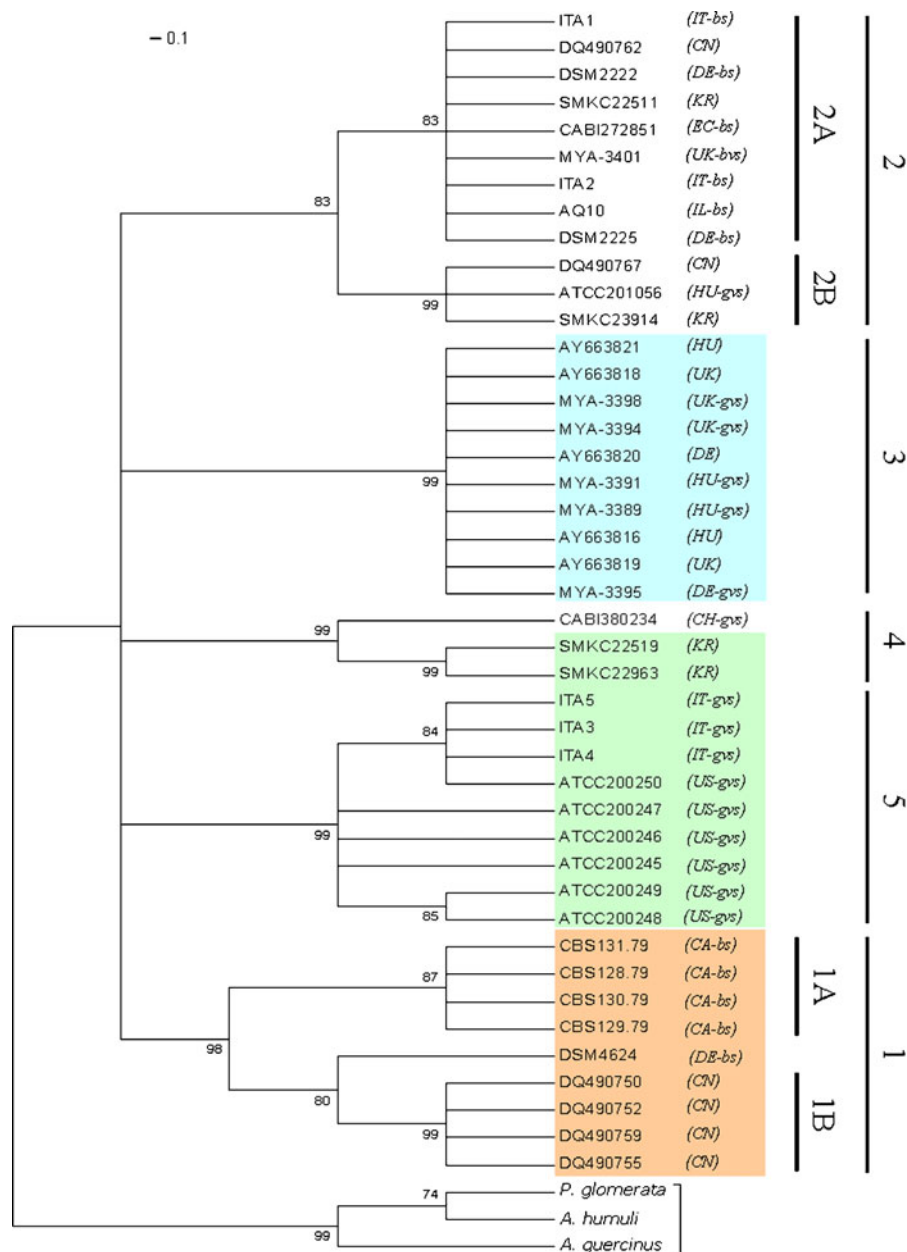


Fig. 4 The maximum-likelihood tree for the ITS rDNA sequences of 43 *Ampelomyces quisqualis* strains. Sequences were inferred with the Mega4 software package, using the Jukes-Cantor substitution model and rate uniformity among sites. The gaps in the 450-character-long alignment were handled as missing characters. The bootstrap values were obtained from maximum-likelihood and maximum-parsimony analyses. The values shown above the branches are percentages of 1000 replicates; scores below 50% are not shown. The scale bar represents 0.1 substitutions per nucleotide position. The five groups (1–5) discussed in the text are indicated on the tree.

Different colors are used to indicate the different powdery mildew host species: *blue* = apple, *green* = grapevine, *orange* = cucumber, no color = other species). In brackets: geographic origin, colony color and growth pattern of each strain. Geographic origin: DE = Germany, UK = United Kingdom, HU = Hungary, CA = Canada, CH = Switzerland, IT = Italy, IL = Israel, EC = Ecuador, CN = China, KR = South Korea, US = United States of America. Colony color and growth pattern: bs = brown, slow-growing; bvs = brown, very slow-growing; gvs = green, very slow-growing)

(clades 4 and 5) with bootstrap (BS) values between 98% and 99% mainly clustered into four different genetic groups. However, different sequence similarity was observed among strains in clade 1 (92–100%), clade 3 (99–100%), clade 4 (95–100%) and clade 5 (97–100%). Strains isolated from apple powdery mildew and classified within clade 3 displayed the least ITS diversity, even though they were collected in three different geographical areas.

Comparing clades, clade 1 containing strains isolated from cucumber had the greatest ITS sequence diversity. In fact, these strains can be divided into two different subclades, the first composed of strains collected in Canada (1A) and the second composed of strains collected in China (1B). The strains within each of these subclades share exactly the same ITS sequence (100%). Comparing strains according to their host, the strains isolated from grapevine powdery mildew clearly displayed the highest level of ITS diversity. These strains fell into two different clades: clade 4, which includes strains isolated in China, and clade 5, which includes strains isolated in the USA and Italy. Taking a closer look at the ITS sequence clustering in clade 5, it is seen that, with the exception of ATCC200250, the Italian strains seem to differ from the American strains. The only ITS clade which includes strains isolated from different mycohosts and in different geographical regions was clade 2. This clade contains two strains from grapevine powdery mildew (ITA1 and ITA2), one strain from cucumber powdery mildew (DSM2222) and nine strains isolated from several different ornamental plants. The 12 strains in clade 2 (BS value of 83%) were further divided into two subgroups: subclade 2A (nine sequences) and subclade 2B (three sequences), which were supported by BS values of 83% and 99%, respectively, and which each had ITS-similarity scores of 98–100%. Among the strains included in subclade 2A, only the ITS sequence of strains DSM2225 and DQ490762 differed from that of the commercial strain AQ10.

Interestingly, strains isolated from the same fungal host and assigned to the same ITS group (clades 1, 3, 4 and 5) showed similar colony morphology and growth characteristics. Strains belonging to clades 3, 4 and 5 are green and very slow-growing, whereas strains belonging to clade 1 are brown and grow more quickly. Again, clade 2 is an exception in that it includes strains that differ in the color of their colonies and their *in vitro* growth rates.

Discussion

Ampelomyces quisqualis strains have been studied mostly for use in the biological control of powdery mildew on different plant species. Data on the morphology and cultural patterns of *A. quisqualis* strains found in the literature are contradictory and incomplete. There is a need for further cultural and morphological investigations aiming at the identification of phenotypic markers that can be used to differentiate genetically distinct groups (Park *et al.* 2010) within the true *A. quisqualis* [*e.g.* Type II (Kiss 1997)].

The aim of this study was to understand whether there are relationships between the origins (host, geographic location) of different strains of *A. quisqualis* and their phylogeny and/or other characteristics (cultural, growth, morphology, physiology), in order to establish the existence of any host/geographic adaptation of this mycoparasite. Our results provide solid evidence for the existence of different physiological forms among slow-growing *A. quisqualis* strains [*e.g.* Type II (Kiss 1997)] isolated from different fungal hosts. These physiological forms vary in terms of their cultural, morphological and physiological characteristics. In the present study, the *A. quisqualis* strains cultured on artificial media did not differ in the dimensions or shape of their fruiting bodies (pycnidia and conidia). Thus, these morphological characters are insufficient for accurate identification of different strains in the laboratory. Based on colony characteristics and growth rates on artificial media, two fairly homogeneous groups can be clearly distinguished: dark-brown, slow-growing strains and olive-green, very slow-growing strains. Thus, a clear relationship was detected between the growth rate and colony color of *A. quisqualis* strains. Moreover, we demonstrated the existence of a relationship between the original fungal host and cultural and growth characteristics. In contrast, we did not identify any relationship between the geographic origins of the different strains and their *in vitro* growth rates.

As expected, temperature greatly affects the growth rate of *A. quisqualis*. We observed differences in the behavior of different strains at different temperatures and six strains (DSM4624, CBS128.79, CBS129.79, CBS130.79, CBS131.79, CABI272851) grew faster than the others at all of the tested temperatures.

Powdery mildew strongly affects the germination of *A. quisqualis* conidia during the recognition phase of parasitism, but specialization is indistinct. In fact, the germination rates and tube elongation of all of the examined *A. quisqualis* strains could be stimulated by all of the examined powdery mildew species and not only by the conidia of their original fungal host. However, we did observe statistically significant differences among the tested strains. Strain ITA3 was most strongly stimulated and, interestingly, among all the tested strains, the commercial strain AQ10 was stimulated the least by the presence of conidia of different powdery mildew species.

It was found that the growth rate of *A. quisqualis* on artificial media is not related to the conidial germination or the germ-tube elongation of *A. quisqualis* during the recognition phase of parasitism. Therefore, we think that a screening program based on *in vitro* growth rates is not the right way to select strains of this mycoparasitic fungus for use in biocontrol programs.

Finally, phylogenetic analysis of the ITS rDNA sequence revealed a high level of genetic diversity among Type II strains of *A. quisqualis* and suggested that ITS groups could be related to the host fungus. In most cases, a degree of mycohost specialization was present in the *A. quisqualis* strains, as demonstrated by the phylogenetic studies conducted by Park *et al.* (2010). Our study revealed that strains isolated from the same host species had similar cultural, morphological and growth characteristics and grouped into the same ITS group. Moreover, among the strains isolated from cucumber powdery mildew and grapevine powdery mildew, we observed differentiation among *A. quisqualis* strains that was congruent with differences in geographic origin. Therefore, it appears that, in some cases, powdery mildew fungi and *A. quisqualis* might have coevolved through the processes of specialization and adaptation without the mycoparasite losing the ability to be stimulated by other hosts.

The present results indicate that the Type II strains of *A. quisqualis* examined in this study are widely scattered among four clades and two subclades identified by Park *et al.* (2010). Furthermore, the presence of an additional clade (5) was revealed. This clade includes the Type II strains isolated from grapevine powdery mildew whose morphology and physiology were analyzed in this experiment, as well

as all of the American strains of grapevine powdery mildew. Clade 5 appears homogeneous in regard to the mycohost (*E. necator*), as well as colony color and growth pattern. However, the clustering of strains within clade 5 may point to a certain tendency toward geographic–genetic differentiation, suggesting possible local adaptations of this mycoparasite that should be verified in wider studies. Clade 2A includes the commercial AQ10 strain, as well as five strains (CABI272851, MYA-3401, ITA1, ITA2, SMK22511) that were isolated after the commercialization of AQ10 and have identical ITS sequences, suggesting that those five strains may have originated from AQ10 treatments applied in the same area. This would indicate that the commercial strain can spread and persist in nature. On the other hand, strain DQ490762 belonging to Clade 2A, which was isolated after the commercialization of AQ10 and having a different nucleotide sequence, probably represents a distinct strain.

Screening is a crucial step in the selection of strains capable of providing highly effective biocontrol. Future studies should attempt to clarify whether the conidial germination rates of *A. quisqualis* strains are related to differences in virulence against powdery mildew, which would be a relevant factor in the selection of biocontrol agents.

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