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No Indication of Strict Host Associations in a Widespread Mycoparasite: Grapevine Powdery Mildew (*Erysiphe necator*) Is Attacked by Phylogenetically Distant *Ampelomyces* Strains in the Field

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ABSTRACT

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Pycnidial fungi belonging to the genus *Ampelomyces* are common intracellular mycoparasites of powdery mildews worldwide. Some strains have already been developed as commercial biocontrol agents (BCAs) of *Erysiphe necator* and other powdery mildew species infecting important crops. One of the basic, and still debated, questions concerning the tritrophic relationships between host plants, powdery mildew fungi, and *Ampelomyces* mycoparasites is whether *Ampelomyces* strains isolated from certain species of the Erysiphales are narrowly specialized to their original mycohosts or are generalist mycoparasites of many powdery mildew fungi. This is also important for the use of *Ampelomyces* strains as BCAs. To understand this relationship, the nuclear ribosomal DNA internal transcribed spacer (ITS) and partial actin gene (*act1*) sequences of 55 *Ampelomyces* strains from *E. necator* were analyzed together with those of 47 strains isolated from other powdery mildew species. These phylogenetic analyses distinguished five major clades and strains from *E. necator* that were present in all but one clade. This work was supplemented with the selection of nine inter-simple sequence repeat (ISSR) markers for strain-specific identification of *Ampelomyces* mycoparasites to monitor the environmental fate of strains applied as BCAs. The genetic distances among strains calculated based on ISSR patterns have also highlighted the genetic diversity of *Ampelomyces* mycoparasites naturally occurring in grapevine powdery mildew. Overall, this work showed that *Ampelomyces* strains isolated from *E. necator* are genetically diverse and there is no indication of strict mycohost associations in these strains. However, these results cannot rule out a certain degree of quantitative association between at least some of the *Ampelomyces* lineages identified in this work and their original mycohosts.

Host specificity has always been a controversial issue in parasites (3,5,32,38). One of the most intriguing questions is the often detected host-specific differentiation in closely related parasites, such as the coexistence of cryptic species, each specialized to one or more hosts. This phenomenon has received special attention in fungal parasites of plants especially when it has become clear that many formally described plant-pathogenic fungi, thought to have broad host ranges, consist, in fact, of sibling/cryptic species that might be specialized to only a few hosts (4,20,33). However, in some groups of fungal plant pathogens genetic differentiation did not always correlate with host range patterns (11,14,46,47). This indicates that narrow host specialization is unlikely to be the single force driving the differentiation of closely related parasites.

In fungal mycoparasites, i.e., fungi parasitizing other fungi, host-driven differentiations are not well understood. This is true even for those mycoparasites that have long been studied and commercially explored as biological control agents (BCAs) of plant pathogens infecting economically important crops. An example for such commercialized mycoparasites with controversial results on their (myco)host specialization is provided by pycnidial fungi belonging to the genus *Ampelomyces* (Ascomycota) (18). These are known to occur in the field exclusively only inside the mycelia of powdery mildew fungi, the Erysiphales, obligate plant parasites. All known *Ampelomyces* spp. are strictly specialized to powdery mildews. Powdery mildew species, in turn, are each specialized to one or a few host plant species (10,44). In fact, these are well defined natural tritrophic relationships (16) where one of the most interesting basic questions is whether *Ampelomyces* strains isolated from certain species of the Erysiphales are narrowly specialized to their original mycohosts or are generalist mycoparasites of many powdery mildew fungi. Understanding this relationship is also important for the use of *Ampelomyces* strains as BCAs against powdery mildews because recent studies explained low field performance of an *Ampelomyces*-based biofungicide in terms of strain-specific differences in mycohost range (28) and variations in the virulence of different strains in certain powdery mildew species (1). However, several other studies did not support such *Ampelomyces*–mycohost associations (17,23,42,43).

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It has repeatedly been shown that *Ampelomyces* strains are genetically diverse based on analyses of nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequences (23,27,40, 42), actin gene (*act1*) sequences (28), and microsatellite genotypes (13,17). Recently, this diversity was explained based on either host specializations (28) or temporal isolation (17). The notion of mycohost specialization was also not supported by most cross-inoculation experiments (23,42,43) which showed that a number of strains isolated from different powdery mildew species were all able to parasitize one or two test powdery mildew species in vitro. Similarly, a field experiment demonstrated that genetically different *Ampelomyces* strains naturally occurring in certain powdery mildew species can easily disperse and parasitize other powdery mildew species nearby on their respective host plants (17). However, an in vitro experiment demonstrated that *Ampelomyces* strains from grapevine powdery mildew (*Erysiphe necator*) parasitized much more intensively their original mycohost species than two other test powdery mildew species (7) and a recent work reported differences in the virulence of some *Ampelomyces* strains against three powdery mildew species (1).

Most studies on *Ampelomyces* dealt with strains isolated from many different powdery mildew species/genera, usually with only one or few strains from each mycohost species (1,2,23,27,28, 40,43). Only two studies investigated a high number of strains isolated from the same powdery mildew species and these led to contrasting results. While a large number of strains isolated from *Arthrocladiella mougeotii* infecting *Lycium halimifolium*, a common solanaceous weed, were genetically diverse based on their rDNA ITS sequences (17), all the strains isolated from apple powdery mildew (*Podosphaera leucotricha*) belonged to a unique rDNA ITS haplotype (42) and were genetically highly differentiated from other strains based on microsatellite analyses (17). The latter result was explained on the basis of isolation in time of *Ampelomyces* populations parasitizing apple powdery mildew from those present in other powdery mildew species because *P. leucotricha* produces epidemics especially in spring while most other powdery mildew species start to become widespread in the same environments mainly in autumn. Thus, the mycohost-driven genetic differentiation of that particular *Ampelomyces* lineage may be the result of differences in mycohost phenology rather than strict specialization to apple powdery mildew (17).

Because the recent controversies on the potential mycohost specialization in *Ampelomyces* were mainly linked to the use of different strains as BCAs of grapevine powdery mildew (1,2), one of the most important pathogens of grapevine worldwide, we chose this mycohost species in the present study to investigate the genetic diversity of *Ampelomyces* strains that naturally occur in the grapevine powdery mildew mycelium. Currently, there is lack of data on the genetic diversity of *Ampelomyces* strains from *E. necator*, which are necessary for molecular identification of strains used as BCAs in vineyards. We hypothesized that the detection of those ITS and *act1 Ampelomyces* haplotypes in *E. necator* that have already been identified in other powdery mildew species would indicate no host-driven differentiation. The specific objectives of this work were to (i) isolate many *Ampelomyces* strains from grapevine powdery mildew in the field, (ii) isolate *Ampelomyces* strains from potted powdery mildew-infected grapevine plants used as 'traps' for mycoparasites in field experiments, (iii) analyze the ITS and the *act1* sequences of the newly isolated strains together with sequence data obtained in previous works, and (iv) select new molecular markers for strain-specific identifications of *Ampelomyces*.

MATERIALS AND METHODS

***Ampelomyces* strains from *E. necator*.** To isolate *Ampelomyces* strains, powdery mildew-infected grapevine leaves were repeatedly collected from two sources: (i) 5 Hungarian and 15 Italian vineyards in autumn 2009 and 2010, and (ii) potted, powdery mildew-infected grapevine plants, 'Chardonnay', used as 'traps' for any airborne *Ampelomyces* inoculum as in similar experiments carried out by Kiss et al. (17). These potted plants were grown from cuttings in the greenhouse until they developed 6 to 10 leaves and then were artificially inoculated with *Ampelomyces*-free *E. necator* inoculum that was maintained on other potted grapevine plants in isolation chambers. These inoculated plants were then placed outdoors at a single site in Budapest, Hungary when sporulating powdery mildew colonies appeared on their leaves. This was done three times using 5 to 10 plants each time in August and September 2009, and trap plants were kept outdoors until leaf fall. Powdery mildew-infected grapevine leaves collected from both vineyards and trap plants were taken to the laboratory and examined for the presence of intracellular pycnidia of *Ampelomyces* in the conidiophores and ascomata (chasmothecia) of *E. necator* under a stereomicroscope. When found, *Ampelomyces* mycoparasites were isolated as described in Liang et al. (23). Information on the newly isolated strains is shown in Table 1. Eleven of these were deposited at Centraalbureau voor Schimmelcultures (CBS), the Netherlands (Table 1). In addition, five *Ampelomyces* strains isolated earlier from *E. necator* in the United States (7) were also included in this work as well as DNA sequence data for two strains isolated from *E. necator* in Korea (28) (Table 2). A few other strains from *E. necator* reported in the literature (1,2) were, however, inaccessible for this work.

Other *Ampelomyces* strains. A total of 47 strains isolated from powdery mildew species other than *E. necator* in earlier studies were also included in this work. For some of these strains, both the ITS and the actin gene sequences were available in GenBank and for other strains DNA sequences were determined in this study (Table 2).

DNA extraction. For all polymerase chain reaction (PCR)-based works, except inter-simple sequence repeat (ISSR) analyses, whole-cell DNA was extracted from 10 to 15 mg of freeze-dried mycelia of the strains, grown for 2 to 3 weeks on Czapek-Dox medium supplemented with 2% malt, using a Qiagen DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA samples were stored at –18°C until use. For ISSR analyses, a rapid DNA extraction was carried out based on a method described by Edwards et al. (6) with some modifications. Fresh mycelium, approximately 100 mg, was dry-grinded, and 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.0; 250 mM NaCl; 125 mM EDTA; and 0.5% sodium dodecyl sulfate) was added and the mixture was shaken for 1 min. The suspension was centrifuged for 5 min at full speed and the supernatant was transferred into a new tube containing 300 µl of isopropanol. DNA was collected by centrifugation for 10 min, washed with 70% ethanol, resuspended in 50 µl of H_2O, and kept at –18°C until use.

PCR amplification and sequencing of the nrDNA ITS region and a part of the actin gene. The nrDNA ITS region was amplified and sequenced using the fungal-specific primer pair ITS1F/ITS4 (9) as described in Szentiványi et al. (42). The Act-1/Act-5ra primer pair (50) was used to amplify an approximately 850-bp-long part of the actin gene. PCRs were carried out as described in Voigt et al. (49) and Park et al. (28). PCR products were directly sequenced with the actin gene primers Act-1, Act-2, and Act-5ra (50).

Each PCR amplification was carried out in parallel in three tubes under identical conditions and the three PCR products were mixed before further use. As described by Kovács et al. (19), the errors that might be generated by DNA polymerases during direct sequencing of PCR products could be practically eliminated by this procedure. This is because the possibility of a DNA polymerase error at the same nucleotide position in three identical PCRs is almost zero.

Phylogenetic analyses of the ITS and actin gene sequences. Electrophoregrams were processed and analyzed with the Staden Program Package (39). The ITS sequences were aligned using MAFFT version 6 (15), while the actin gene sequence alignment was completed with PRANK (25) using the PRANKSTER interface. The final alignment was 517 characters long for the ITS and 798 characters long for the actin gene data set. The ITS and the actin gene sequences of *Phoma herbarum* strain CBS 567.63 (GenBank accession nos. JF810528 and AY748978) served as outgroups. The best-fit nucleotide substitution models were selected with jModelTest 0.1.1 (31) using Akaike information criterion (AIC). Maximum likelihood (ML) phylogenetic analyses were carried out with the online version of PHYML 3.0 (12). The GTR nucleotide substitution model was used with the ML estimation of base frequencies for ITS and the TN93 model for the actin gene data set. Six substitution rate categories were set and the gamma distribution parameter and the proportion of invariable sites were estimated. The statistical supports of the branches were tested by ML bootstrap analyses with 1,000 replicates. In addition, Bayesian (MCMC) analysis was performed with both ITS and actin gene data sets with MrBayes 3.1.2 (34) using GTR nucleotide substitution model with the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/index.aspx). Markov chains were run over 5,000,000 generations; one tree was sampled every 100 generations with a burn-in at 7,500 trees. Phylogenetic trees were viewed and edited by Tree Explorer of the MEGA 4 program (45) and a text editor. The ITS and actin gene sequences determined in this work were deposited in GenBank (Tables 1 and 2).

The congruence between the ITS and the actin gene sequence alignments was assessed by the approximately unbiased test (35) as implemented in CONSEL (36). The ML trees used in this test were estimated in 10 replicates and site-wise likelihoods were

TABLE 1. Designations, place and date of collection, and internal transcribed spacer (ITS) and actin sequence database accession numbers of *Ampelomyces* strains isolated from *Erysiphe necator* in this study[a]

Strain designation[b]	Place of collection	Date of collection	GenBank accession number	
			ITS	Actin gene
Vitis1	Canneto Pavese, Italy	5 Oct. 2009	JN417710	JN621818
Vitis9	Site 1, Piacenza, Italy	5 Oct. 2009	JN417711	JN621819
Vitis11	Site 1, Piacenza, Italy	5 Oct. 2009	JN417712	JN621820
Vitis12	Site 1, Piacenza, Italy	5 Oct. 2009	JN417713	JN621821
Vitis15 (CBS 132347)	Site 1, Piacenza, Italy	5 Oct. 2009	JN417714	JN621822
Vitis21	Site 1, Travazzano, Italy	8 Oct. 2009	JN417715	JN621823
Vitis23	Site 1, Travazzano, Italy	8 Oct. 2009	JN417716	JN621824
Vitis25	Site 1, Travazzano, Italy	8 Oct. 2009	JN417717	JN621825
Vitis26	Site 2, Travazzano, Italy	8 Oct. 2009	JN417718	JN621826
Vitis30	Site 2, Travazzano, Italy	9 Oct. 2009	JN417719	JN621827
Vitis32	Pieve Dugliara, Italy	12 Oct. 2009	JN417720	JN621828
Vitis34	Pieve Dugliara, Italy	12 Oct. 2009	JN417721	JN621829
Vitis35	Site 2, Piacenza, Italy	7 Oct. 2009	JN417722	JN621830
Vitis38	Site 1, Paterno, Italy	15 Oct. 2009	JN417723	JN621831
Vitis39	Site 2, Paterno, Italy	14 Oct. 2009	JN417724	JN621832
Vitis42	Site 2, Paterno, Italy	14 Oct. 2009	JN417725	JN621833
Vitis44	Site 3, Paterno, Italy	15 Oct. 2009	JN417726	JN621834
Vitis45	Site 3, Paterno, Italy	15 Oct. 2009	JN417727	JN621835
Vitis46	Grugliasco, Italy	15 Oct. 2009	JN417728	JN621836
Vitis49	Grugliasco, Italy	15 Oct. 2009	JN417729	JN621837
Vitis50	Portici, Italy	15 Oct. 2009	JN417730	JN621838
Vitis51	Santo Stefano, Italy	15 Oct. 2009	JN417731	JN621839
Vitis55	Santo Stefano, Italy	15 Oct. 2009	JN417732	JN621840
Vitis56	Santo Stefano, Italy	17 Oct. 2009	JN417733	JN621841
Vitis60	Santo Stefano, Italy	17 Oct. 2009	JN417734	JN621842
Vitis61	Piagge, Italy	16 Oct. 2009	JN417735	JN621843
Vitis66	Piagge, Italy	16 Oct. 2009	JN417736	JN621844
Vitis68	Jesi, Italy	16 Oct. 2009	JN417737	JN621845
Vitis69 (CBS 132219)	Jesi, Italy	16 Oct. 2009	JN417738	JN621846
Vitis70 (CBS 132220)	Jesi, Italy	16 Oct. 2009	JN417739	JN621847
Vitis72	Portonovo, Italy	16 Oct. 2009	JN417740	JN621848
Vitis75	Portonovo, Italy	16 Oct. 2009	JN417741	JN621849
Vitis76	Portonovo, Italy	16 Oct. 2009	JN417742	JN621850
Vitis79	Portonovo, Italy	16 Oct. 2009	JN417743	JN621851
Vitis81	Site 1, Szekszárd, Hungary	22 Oct. 2009	JN417744	JN621852
Vitis83	Site 1, Szekszárd, Hungary	22 Oct. 2009	JN417745	JN621853
Vitis91	Site 2, Szekszárd, Hungary	22 Oct. 2009	JN417746	JN621854
Vitis94	Site 3, Szekszárd, Hungary	22 Oct. 2009	JN417747	JN621855
Vitis98*	Budapest, Hungary	28 Sept. 2009	JN417748	JN621856
Vitis101* (CBS 132221)	Budapest, Hungary	15 Sept. 2009	JN417749	JN621857
Vitis102* (CBS 132222)	Budapest, Hungary	15 Sept. 2009	JN417750	JN621858
Vitis105* (CBS 132223)	Budapest, Hungary	15 Sept. 2009	JN417751	JN621859
Vitis107 (CBS 132224)	Eger, Hungary	22 Sept. 2009	JN417752	JN621860
Vitis109 (CBS 132225)	Eger, Hungary	22 Sept. 2009	JN417753	JN621861
Vitis113 (CBS 132346)	Eger, Hungary	22 Sept. 2009	JN417754	JN621862
Vitis114	Eger, Hungary	22 Sept. 2009	JN417755	JN621863
Vitis115 (CBS 132226)	Eger, Hungary	22 Sept. 2009	JN417756	JN621864
Vitis117 (CBS 132227)	Eger, Hungary	22 Sept. 2009	JN417757	JN621865

[a] All DNA sequences reported in the table were determined in this work. Whenever strains were isolated in more than one site within a locality, the site number is also shown.

[b] * indicates strain isolated from potted powdery mildew-infected grapevine plants exposed outdoors as traps for *Ampelomyces*.

computed in PhyML 3.0 (12). P values ≤ 0.05 were considered indicative of significant conflict of single locus trees.

ISSR analysis. Nonanchored ISSR primers were selected based on the simple sequence repeat (SSR) motif abundance in fungi reported by Lim et al. (24). Nine out of the eleven ISSR primers screened were selected for this work based on production of polymorphic patterns and reproducibility and readability of PCR products (Table 3). All ISSR PCR amplifications were done in a final volume of 25 µl with the following components: GreenTaq (Fermentas) 12.5 µl, 50 ng of template DNA, 1 µM primer, and water. PCR parameters were as follows: an initial denaturation step at 95°C for 2 min, followed by 35 cycles each consisting of 30 s at 90°C for denaturation, then 40 s at 45, 50, 60, or 72°C, depending on the ISSR primers (Table 3), for primer annealing, and 1 min at 72°C for extension, and a final extension cycle at 72°C for 5 min. A negative control that lacked template DNA was included for each set of PCR amplifications. All amplifications were carried out at least twice to verify reproduci-

TABLE 2. Designations, place and year of collection, and internal transcribed spacer (ITS) and actin sequence database accession numbers of *Ampelomyces* strains obtained in earlier studies and included in this work[a]

Strain designation	Host fungal species	Host plant species	Year of collection	Place of collection	GenBank accession number	
					ITS	Actin gene
A1 (ATCC 201056)	*Arthrocladiella mougeotii*	*Lycium halimifolium*	1990	Budapest, Hungary	AF035780	JN621873
A8	*A. mougeotii*	*L. halimifolium*	2007	Budapest, Hungary	HM124894	JN621874
A9	*A. mougeotii*	*L. halimifolium*	2007	Budapest, Hungary	HM124895	JN621875
A17	*A. mougeotii*	*L. halimifolium*	2007	Budapest, Hungary	HM124901	JN621876
A62-b	*A. mougeotii*	*L. halimifolium*	2007	Páty, Hungary	HM124924	JN621877
A97	*A. mougeotii*	*L. halimifolium*	2007	Biatorbágy, Hungary	HM124938	JN621878
A98	*A. mougeotii*	*L. halimifolium*	2007	Biatorbágy, Hungary	HM124939	JN621879
A115	*A. mougeotii*	*L. halimifolium*	2007	Budapest, Hungary	HM124955	JN621880
AQ10	*Oidium* sp.	*Catha edulis*	?*	Israel	AF035783	GU330010
B22 (MYA-3392)	*Podosphaera leucotricha*	*Malus domestica*	2002	Prague, Czech Republic	JN417759	JN621881
B34 (MYA-3396)	*P. leucotricha*	*M. domestica*	2002	Cambridge, UK	AY663818	JN621882
B71	*P. leucotricha*	*M. domestica*	2008	Cambridge, UK	JN417760	JN621883
B78	*P. leucotricha*	*M. domestica*	2008	Cambridge, UK	JN417761	JN621884
B91	*P. leucotricha*	*M. domestica*	2008	Canterbury, UK	JN417762	JN621885
B100	*P. leucotricha*	*M. domestica*	2008	Canterbury, UK	JN417763	JN621886
B102	*P. leucotricha*	*M. domestica*	2008	East Malling, UK	JN417764	JN621887
B124-a	*P. leucotricha*	*M. domestica*	2008	Eperjeske, Hungary	HM124964	JN621888
B133	*P. leucotricha*	*M. domestica*	2008	Eperjeske, Hungary	JN417765	JN621889
B226-a	*P. leucotricha*	*M. domestica*	2008	Gotheron, France	JN417766	JN621890
DSM2222	*P. xanthii*	*Cucumis* sp.	?*	Germany	U82450	JN621871
G2	*Erysiphe polygoni*	*Rumex patientia*	2002	Budapest, Hungary	DQ490770	JN621891
G273 (ATCC 200245)	*E. necator*	*Vitis* sp.	1989	USA	HM125018	JN621870
SF414 (ATCC 200246)	*E. necator*	*V. riparia*	1991	USA	HM125015	JN621866
SF418 (ATCC 200247)	*E. necator*	*V. riparia*	1991	USA	JN417758	JN621867
SF419 (ATCC 200248)	*E. necator*	*V. riparia*	1991	USA	HM125016	JN621868
SF423 (ATCC 200250)	*E. necator*	*V. riparia*	1991	USA	HM125017	JN621869
HMLAC226	*E. polygoni*	*Polygonum aviculare*	2003	Mengyin, Shandong, China	DQ490766	JN621872
LV2-b	*Erysiphe* sp.	*Ligustrum vulgare*	2007	Budakeszi, Hungary	HM124990	JN621892
MA6-b	*E. berberidis*	*Mahonia aquifolium*	2007	Budapest, Hungary	HM124996	JN621893
MA8	*E. berberidis*	*Mahonia aquifolium*	2007	Budapest, Hungary	HM124997	JN621894
RS1-a	*P. pannosa*	*Rosa* sp.	2007	Budapest, Hungary	HM125010	JN621896
TP1	*E. trifolii*	*Trifolium pratense*	2007	Gotheron, Hungary	HM125019	JN621895
SMKC22055	*Oidium* sp.	*Cassia nomame*	2006	Namyangju, Korea	GQ324063	GU330007
SMKC22061	*Golovinomyces cichoracearum*	*Achillea sibirica*	2006	Seoul, Korea	GQ324122	GU330004
SMKC22168	*E. sedi*	*Sedum sarmentosum*	2006	Chuncheon, Korea	GQ324118	GQ324191
SMKC22210	*G. cichoracearum*	*Rudbeckia laciniata* var. *hortensis*	2006	Chuncheon, Korea	GQ324113	GQ324181
SMKC22216	*P. pannosa*	*Rosa hybrida*	2006	Hongcheon, Korea	GQ324121	GQ324203
SMKC22264	*P. fusca*	*Erigeron canadensis*	2006	Yanggu, Korea	GQ324033	GQ324152
SMKC22285	*E. cruciferarum*	*Chelidonium majus* var. *asiaticum*	2006	Seoul, Korea	GQ324145	GQ324216
SMKC22286	*E. alphitoides*	*Quercus* sp.	2006	Seoul, Korea	GQ324142	GQ324213
SMKC22313	*E. paeoniae*	*Paeonia lactiflora* var. *hortensis*	2006	Namyangju, Korea	GQ324096	GQ324194
SMKC22334	*E. alphitoides*	*Quercus* sp.	2006	Seoul, Korea	GQ324143	GQ324214
SMKC22341	*E. hommae*	*Elsholtzia ciliata*	2006	Seoul, Korea	GQ324092	GQ324189
SMKC22381	*E. glycines*	*Amphicarpaea edgeworthii* var. *trisperma*	2006	Masan, Korea	GQ324061	GU330006
SMKC22383	*E. hommae*	*E. ciliata*	2006	Masan, Korea	GQ324130	GQ324207
SMKC22455	*P. fusca*	*Youngia japonica*	2006	Seogwipo, Korea	GQ324036	GQ324155
SMKC22470	*P. fusca*	*Taraxacum* sp.	2006	Suwon, Korea	GQ324044	GQ324157
SMKC22472	*E. verniciferae*	*Cotinus coggygria*	2006	Suwon, Korea	GQ324138	GQ324211
SMKC22477	*P. sparsa*	*Metaplexis japonica*	2006	Suwon, Korea	GQ324070	GQ324172
SMKC22478	*G. artemisiae*	*Artemisia feddei*	2006	Suwon, Korea	GQ324088	GQ324183
SMKC22513	*P. fusca*	*Trichosanthes kirilowii*	2006	Seogwipo, Korea	GQ324053	GQ324162
SMKC22519	*E. necator*	*V. flexuosa*	2006	Jeju, Korea	GQ324144	GQ324215
SMKC22963	*E. necator*	*V. vinifera*	2007	Seoul, Korea	GQ324149	GQ324218
SMKC23812	*P. euphorbiae*	*Euphorbia jolkini*	2008	Jinju, Korea	GU329995	GU330011

[a] The identity of the host fungi and host plants of the isolates were determined by their suppliers. DNA sequences determined in this study are underlined. * indicates missing data.

bility of the PCR products separated by electrophoresis in 2% agarose gels and then visualized and photographed under ultraviolet light using a UVP Gel Doc It system.

ISSR markers were considered as dominant markers and the presence or absence of bands with the same size was scored by eye. Only well reproducible bands from 150 to 2,000 bp were taken into consideration. A dendrogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA) method with TREECON (48) based on the coefficient of Nei and Li (26). An additional UPGMA analysis was performed with the SYN-TAX 2000 software package (30) based on Jaccard's coefficient.

RESULTS

Genetic diversity of *Ampelomyces* strains isolated from *E. necator* from the field and trap plants. A total of 44 *Ampelomyces* strains were newly isolated in this work from *E. necator* in Hungary and Italy. All but one of these strains were collected in abandoned vineyards, private gardens, and other places without regular fungicide applications. Only a single strain, Vitis1, came from a fungicide-treated vineyard situated in Canneto Pavese, Italy. Four other strains were isolated from potted and *E. necator*-infected grapevine plants used as traps for mycoparasites (Table 1). The analysis of ITS sequences of these newly isolated 48 strains together with those of seven other strains isolated earlier from grapevine powdery mildew in the United States (7) and Korea (28) and those of 47 *Ampelomyces* strains isolated from other powdery mildew species (Table 2) distinguished five major clades (Fig. 1). Strains isolated from *E. necator* in the field were present in all but one clade of the ITS tree. Those trapped by potted plants were also genetically different belonging to clades 1 and 5. The *act1* sequence analysis distinguished additional groups within the same 102 strains because strains belonging to clade 1 of the ITS tree (Fig. 1) were further divided into seven subclades, 1A to 1G, of the *act1* tree (Fig. 2). All but two strains representing the other four clades of the ITS tree were included in the same clades based on their *act1* sequences (Fig. 2). No conflict was detected ($P \leq 0.05$) between the phylogenies inferred from the ITS and *act1* alignments. The analysis of the combined ITS and *act1* data set resulted in the same grouping of the strains (Supplemental Figure 1).

The two strains which did not cluster in the same ITS and *act1* clades were Vitis117 and SMKC22216; both belonged to ITS clade 2 and *act1* subclade 1D. Thus, *act1* analysis has also revealed that *Ampelomyces* strains from *E. necator* are genetically diverse belonging to all but one major clade. Clade 3, which did not include any strains from *E. necator*, consisted of eight strains isolated from different powdery mildews in Korea by Park et al. (28). Geographic origin of the strains isolated from *E. necator* did not show any clear connection with their grouping according to ITS and/or *act1* sequences. For example, in clade 5, these strains came from Hungary, Italy, and the United States and the two *E. necator* strains from Korea, SMKC22519 and SMKC22963, clustered together with two Hungarian strains in clade 4. There was a single clade, number 5, which did not include any strains isolated from mycohosts other than grapevine powdery mildew. However, when additional strains were included in the ITS analysis, two American strains, AQ2 and AQ3, isolated by Sullivan and White (40) from *E. penicillata* infecting *Platanus occidentalis*, also clustered in this clade (Supplemental Figure 2), which therefore, cannot be considered as a group of grapevine powdery mildew strains only.

ISSR profiles were unique in all of the 54 strains included in the ISSR work, including 44 isolated from *E. necator* (Fig. 3). As expected, the grouping based on ISSR patterns did not always correspond to the phylogenetic relationships revealed by ITS and *act1* analyses but the genetic distances among strains (Fig. 3) have highlighted the genetic diversity of *Ampelomyces* mycoparasites naturally occurring in grapevine powdery mildew.

Strain-specific identification of *Ampelomyces* mycoparasites based on ISSR profiles. Among 11 ISSR primers tested, selected based on the SSR motif abundance in fungi (24), nine produced polymorphic and well detectable bands in the range of 150 to 2,000 bp (Table 3). The multilocus ISSR profiles were different in all of the 54 strains included in the ISSR work (Fig. 3). The geographical and/or the mycohost origin of the strains did not always explain the similarities in the ISSR patterns. For example, only minor differences were detected between strains Vitis115 and Vitis117, both isolated from *E. necator* on the same day and from the same place, and also between strains TP1 and MA6-b isolated from different powdery mildew species in France and Hungary, respectively (Table 2). A total of 35 of the 54 strains tested belonged to the ITS and actin gene clade 1, thus were phylogenetically closely related, but produced distinct ISSR patterns. The commercial *Ampelomyces* strain M-10 from the AQ10 Biofungicide product, belonging to the ITS and actin gene clade 1, was distinguished from all the other strains tested based on multilocus ISSR profiles.

DISCUSSION

No indication of strict host associations in *Ampelomyces* strains isolated from *E. necator*. Recently, two comprehensive studies examined the genetic diversity in *Ampelomyces* mycoparasites isolated from many different powdery mildew species and these led to contrasting results. Park et al. (28) concluded that mycohost specializations explain this diversity, while Kiss et al. (17) suggested that the genetic differentiation of a particular *Ampelomyces* lineage is the result of differences in mycohost phenology rather than strict mycohost specialization. To investigate this issue, we focused on *Ampelomyces* strains present in grapevine powdery mildew in the field and hypothesized that if *E. necator* was parasitized in the field by ITS and *act1 Ampelomyces* haplotypes that have already been identified in other powdery mildew species this would indicate no mycohost specialization. Both the ITS and the *act1* sequence analyses showed that the 55 strains from *E. necator* included in this work represent several distinct phylogenetic lineages within *Ampelomyces* which are not uniquely associated with *E. necator* or any other powdery mildew

TABLE 3. Patterns of inter-simple sequence repeat markers used in this work

Primer code	Primer sequence (5′→3′)	Number of scorable bands (ranging from 150 to 2,000 bp)	PCR T_A (°C)
AMP1	AGAGAGAGAGAGAGAG	4	45
AMP2	ACACACACACACACAC	5	45
AMP3	CCGCCGCCGCCGCCGCCG	5	72
AMP4	ACGACGACGACGACGACG	5	60
AMP5	AGGAGGAGGAGGAGGAGG	4	60
AMP6	AGCAGCAGCAGCAGCAGC	4	72
AMP7	ATCATCATCATCATCATC	5	50
AMP8	AACAACAACAACAACAAC	5	50
AMP9	AAGAAGAAGAAGAAGAAG	4	50

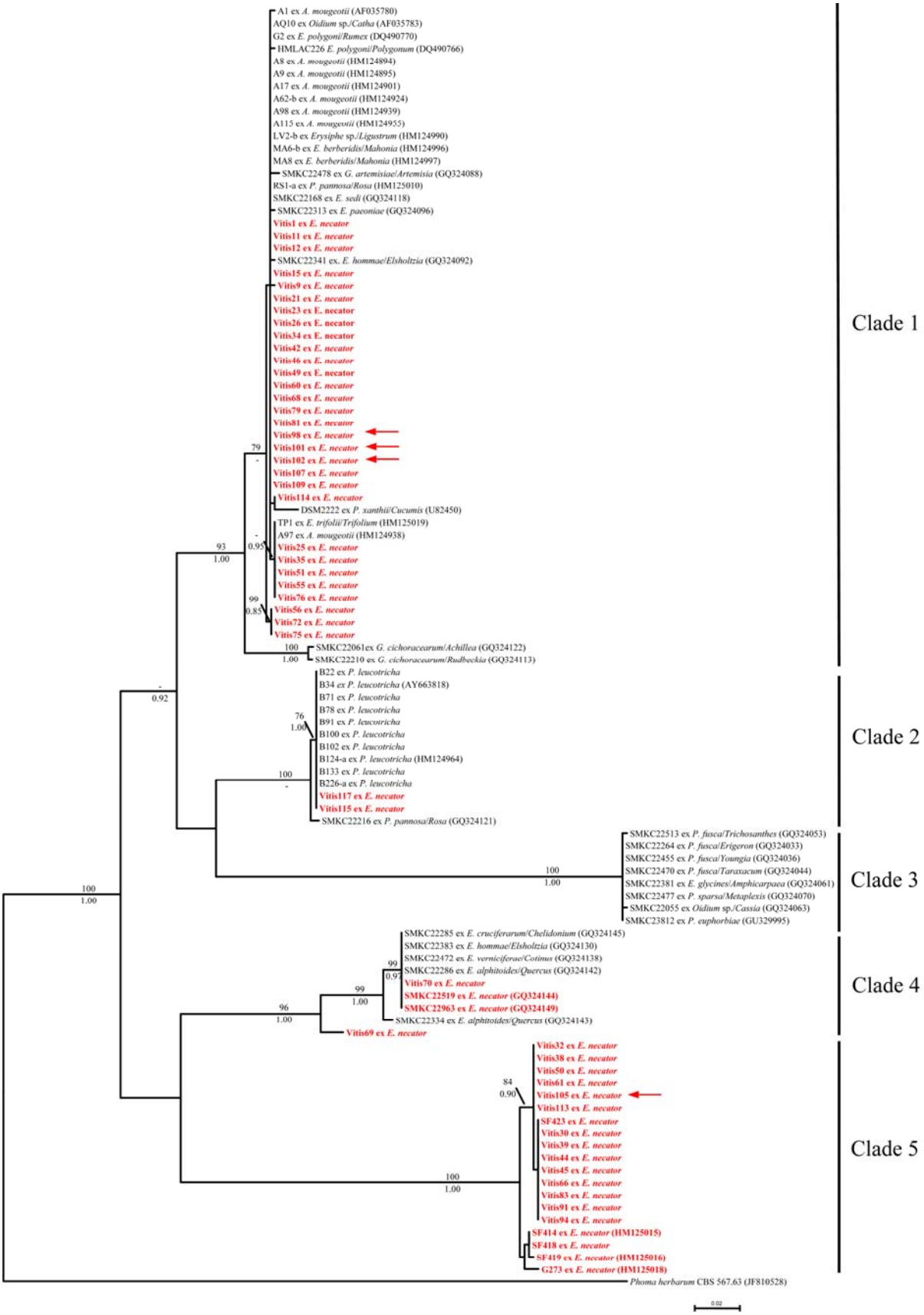

Fig. 1. Maximum likelihood tree based on rDNA internal transcribed spacer (ITS) sequences of 102 *Ampelomyces* strains. The ITS sequence of *Phoma herbarum* CBS 567.63 was used as outgroup. The bootstrap values presented as percentages are above while posterior probabilities are below the branches. Bootstrap values below 70% and posterior probabilities below 0.90 are not shown. The data set comprised 517 characters. Strains isolated from *Erysiphe necator* are shown in red. Arrows point to the strains isolated from potted powdery mildew-infected grapevine plants placed outdoors as traps for *Ampelomyces*. Whenever the same powdery mildew species was collected from more than one host plant species/genera, the host plant genus is also shown. The GenBank accession numbers of the 54 sequences obtained from earlier studies are shown in parentheses, while the accession numbers of the newly determined sequences are included in Tables 1 and 2. Bar indicates 0.02 expected change per site per branch.

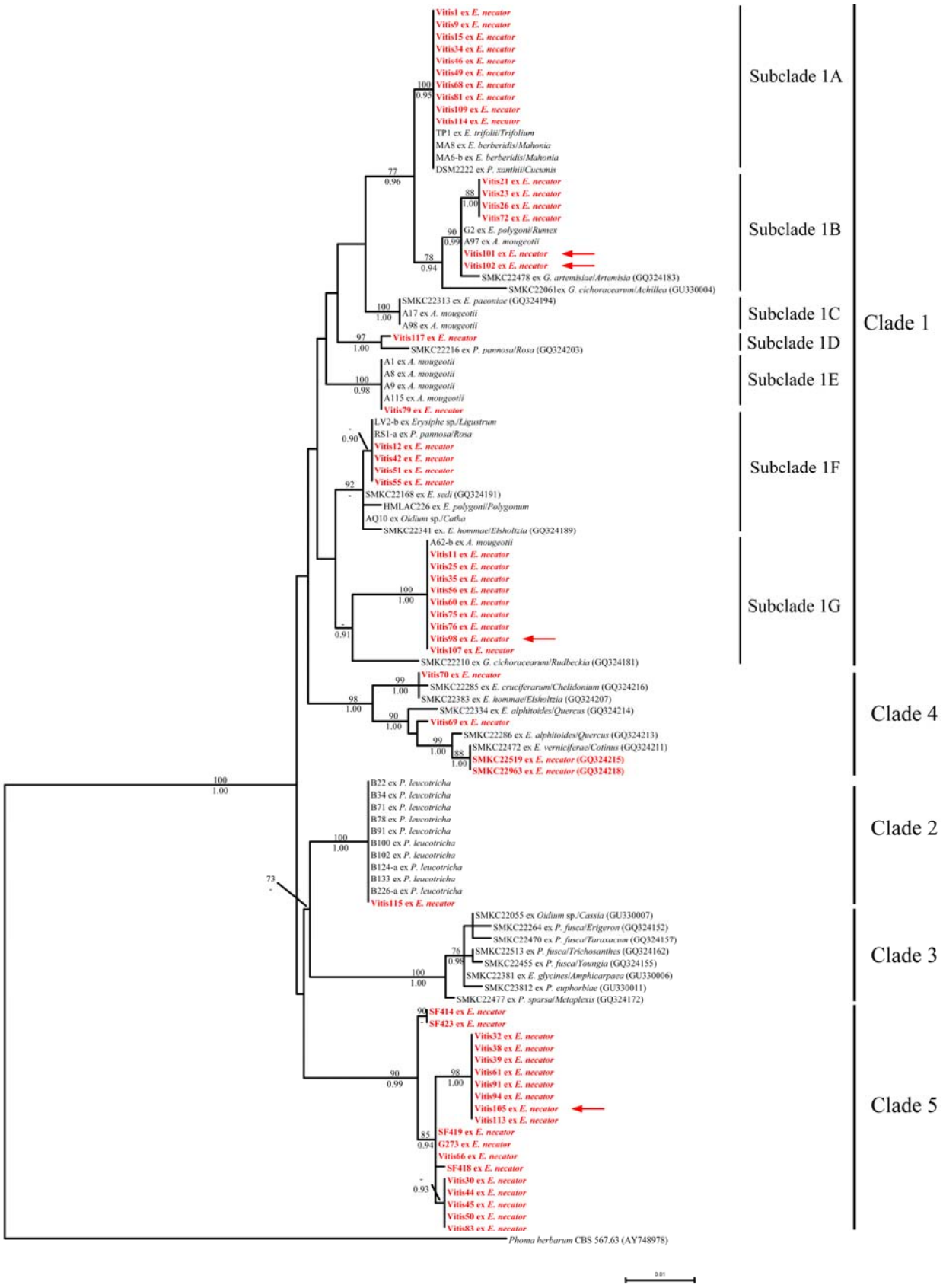

Fig. 2. Phylogenetic tree based on partial actin gene (*act1*) sequences of 102 *Ampelomyces* strains. The partial *act1* sequence of *Phoma herbarum* strain CBS 567.63 served as outgroup. Numbers above the branches denote bootstrap values from 1,000 replications. Percentage values below branches are posterior probabilities. Bootstrap values below 70% and posterior probabilities below 0.90 are not shown. The data set comprised 798 characters. *Ampelomyces* strains isolated from *Erysiphe necator* are shown in red boldface. Arrows point to the strains isolated from potted powdery mildew-infected grapevine plants used as traps for airborne *Ampelomyces* inocula. Accession numbers of *act1* sequences determined in earlier works are shown in parentheses while accession numbers of the newly determined sequences are included in Tables 1 and 2. Whenever the same powdery mildew species was collected from more than one host plant species/genera, the host plant genus is also shown. The clades were numbered according to the clade numbers used on the internal transcribed spacer tree (Fig. 1). Bar indicates 0.01 expected change per site per branch.

species. Similar results were obtained earlier based on a preliminary analysis of ITS sequences of five *Ampelomyces* strains isolated from *E. necator* in Italy (2) but no other information was available for the genetic diversity of *Ampelomyces* mycoparasites occurring naturally in grapevine powdery mildew. In another study, many strains isolated from a common powdery mildew fungus, *A. mougeotii*, also represented three distinct ITS haplotypes, and thus did not show any host association patterns (17). The only clear association of a particular ITS haplotype of *Ampelomyces* with a powdery mildew species, namely *P. leucotricha* infecting apple, was explained based on temporal isolation driven by host plant phenology (17,42).

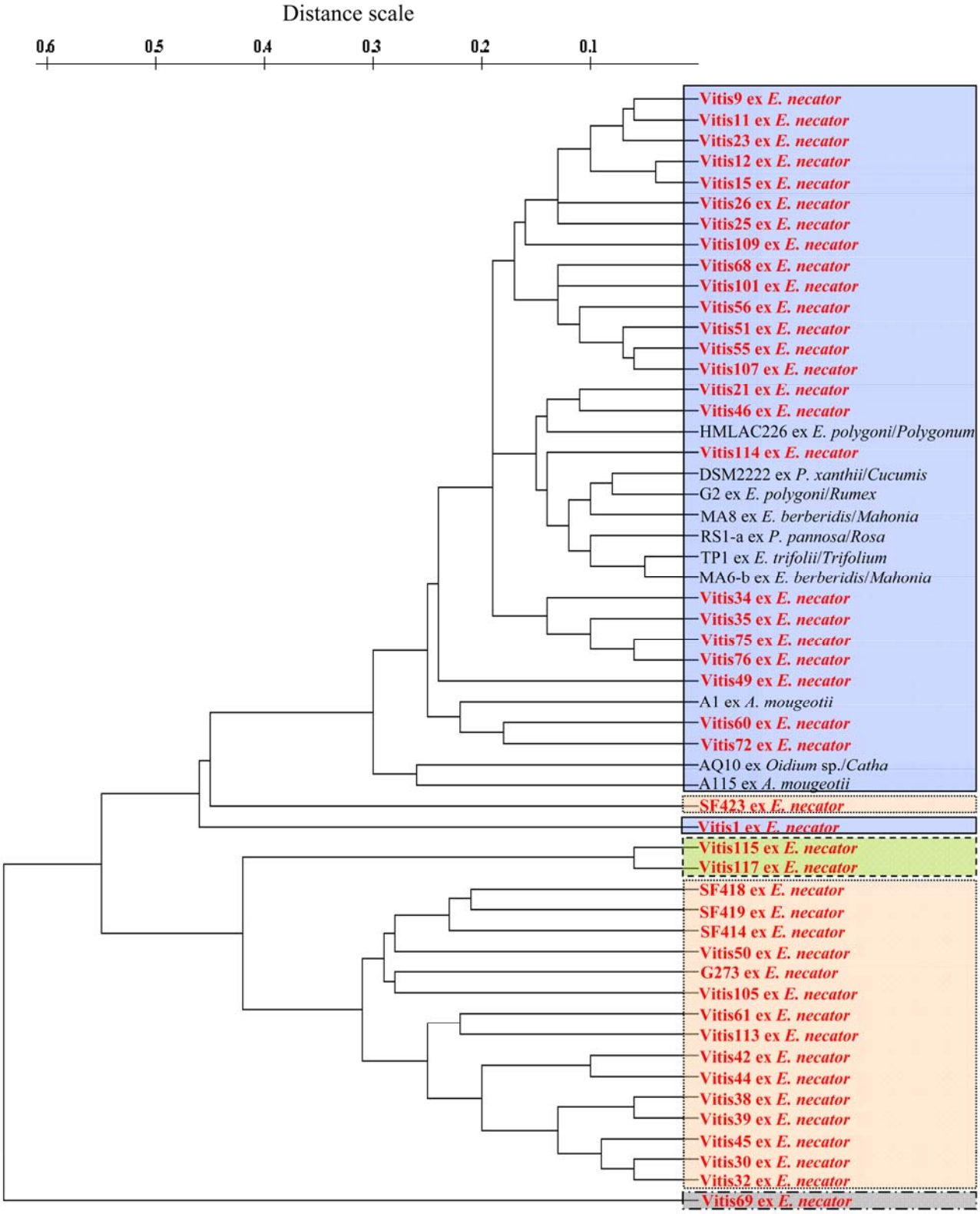

Fig. 3. Unweighted pair-group method with arithmetic average dendrogram showing the clustering pattern of 54 *Ampelomyces* strains, based on their inter-simple sequence repeats (ISSR) patterns determined using nine ISSR markers included in Table 3. The host plant and host fungal species and places and dates of isolations of the *Ampelomyces* strains are shown in Tables 1 and 2. The same background color indicates strains belonging to the same clades of the ITS tree (Fig. 1). Strains isolated from *Erysiphe necator* are shown in red.

Trapping *Ampelomyces* mycoparasites by exposing *E. necator* on potted grapevine plants to any airborne *Ampelomyces* inoculum was a small-scale experiment in this work. However, the results supported the genetic diversity of strains that are able to quickly establish in grapevine powdery mildew. The four trapped strains were diverse based on both ITS and actin gene sequences.

Although our results did not indicate any strict association between particular *Ampelomyces* lineages and grapevine powdery mildew, some degree of genetic differentiation caused by mycohost species and/or host plant species cannot be ruled out in these specialized tritrophic interactions. Clade 5, recognized in both ITS and *act1* analyses, only consisted of strains isolated from *E. necator*, in Europe and the United States, and these strains remained overrepresented in this clade even when many more strains were included in a subsequent analysis of ITS sequences. Earlier data for the mycoparasitic activities of three *E. necator*-derived strains clustered in clade 5, namely G273, SF419, and SF423, suggested that these are more associated with their original mycohost than with two other powdery mildew species used in laboratory experiments. However, no narrow mycohost specialization was found in these three, or any other, *Ampelomyces* strains. For example, Falk et al. (7) showed that strains G273, SF419, and SF423 parasitized cucurbit and strawberry powdery mildews, as well, albeit much less intensively than grapevine powdery mildew. Similar differences in the virulence of some other *Ampelomyces* strains were reported by Angeli et al. (1). In contrast, a field experiment did not reveal significant differences in the mycoparasitic activities of a number of genetically different strains in two test powdery mildew species compared with the values determined in their original mycohosts (17).

Our results are not contradictory to the data indicating a certain degree of association between different *Ampelomyces* strains and their original mycohosts (1,7,28). The mechanisms explaining the diversification in *Ampelomyces* are not well understood and, as suggested recently by Park et al. (28), mycohost-driven differentiations may have indeed contributed to this process, which however, did not lead to strict host specializations in these mycoparasites.

Occurrence of *Ampelomyces* in fungicide-treated vineyards. Most of our trials to detect and isolate *Ampelomyces* from powdery mildewed grapevine leaves collected from vineyards treated regularly with fungicides were unsuccessful (data not shown) and only 1 out of the 44 strains newly isolated in this work came from a fungicide-treated vineyard. Another work reported that the percentage of parasitized *E. necator* chasmothecia was generally higher in abandoned vineyards than in treated ones although the data on the occurrence of *Ampelomyces* in these two types of vineyard were not compared (8). In a recent Italian survey, such a comparison was not performed because of the limited number of samples (2). Such data would be interesting because it was repeatedly shown that *Ampelomyces* can tolerate various fungicide applications (29,37,41,43); however, our sampling experience in Hungarian and Italian vineyards suggested otherwise.

ITS and actin gene sequences are useful markers to distinguish cryptic species in *Ampelomyces*. The taxonomy of the genus *Ampelomyces* is still controversial and a well-founded species concept and species recognition are needed for this genus (18). ITS sequence analyses have already identified a number of phylogenetically distinct groups (23,28,42) and recently Park et al. (28) showed that *act1* sequences are also useful markers in distinguishing *Ampelomyces* lineages. This work expanded that study by analyzing the ITS and *act1* sequences in t38 same strains and revealing that the same lineages are obtained based on the phylogenetic analysis of these two loci albeit actin gene sequences reveal more variation in *Ampelomyces*.

ISSR markers are useful for strain-specific identification of *Ampelomyces* mycoparasites. The nine ISSR markers used in this work represent the first tool to distinguish individual *Ampelomyces* strains. Six polymorphic microsatellite markers (13) were also useful for this purpose but Kiss et al. (17) showed that those do not amplify in most strains isolated from powdery mildews other than *P. leucotricha*. The ISSR markers clearly distinguished all the strains included in this work, although most of them belonged to the same lineage according to phylogenetic analyses. Consequently, these markers could be used to monitor the environmental fate of strains applied as BCAs such as the commercial AQ10 strain and a few others which have been developed more recently for this purpose (21,22).

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