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First Report of an Emerging and Destructive Powdery Mildew Agent Erysiphe corylacearum in Hazelnut in Turkey

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Corresponding Author:	Arzu Sezer, Ph.D Hazelnut Research Institute Giresun, TURKEY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Hazelnut Research Institute
Corresponding Author's Secondary Institution:	
First Author:	Arzu Sezer, Ph.D
First Author Secondary Information:	
Order of Authors:	Arzu Sezer, Ph.D
	F. Sara Dolar, Prof.
	S. James Lucas, Ph.D
	Çiğdem Köse, B.Sc
	Ebru Gümüş, M.S
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Hazelnut (Corylus avellana L.) is Turkey's most valuable agricultural export, and an essential source of income for many families in the Black Sea Region. In spring 2013, hazelnut leaves, fruit clusters and shoots showing powdery mildew infection symptoms different from those observed previously were discovered in Giresun, Ordu and Trabzon provinces of Turkey. The disease has become epidemic throughout all hazelnut production areas spreading from east to west of the Black Sea Region over the subsequent. This new and highly destructive powdery mildew agent has been identified as Erysiphe corylacearum U. Braun & S. Takam. based on its morphological characteristics and DNA sequence of the internal transcribed spacer (ITS) and 28S regions of the ribosomal DNA. Its pathogenicity to this species has been examined in an infection test and proven for the first time. To our knowledge, this is the first report of E. corylacearum parasitization of Corylus avellana worldwide.
Suggested Reviewers:	Uwe Braun, Prof. Martin-Luther-Universitat Halle-Wittenberg uwe.braun@botanik.uni-halle.de He has a distinguished career in mycology, fungal systematic research, especially about on powdery mildews, known worldwide
	Gürsel Karaca, Prof Suleyman Demirel Universitesi Ziraat Fakültesi gurselkaraca@sdu.edu.tr She works on phytopathology, mycology and have some studies on hazelnut fungal diseases.
	Nuray Özer, Prof

	Namık Kemal University Faculty of Agriculture nozer@nku.edu.tr She works on Plant Pathology, mycology and has some studies on obligate fungal plant pathogens.

colour figure

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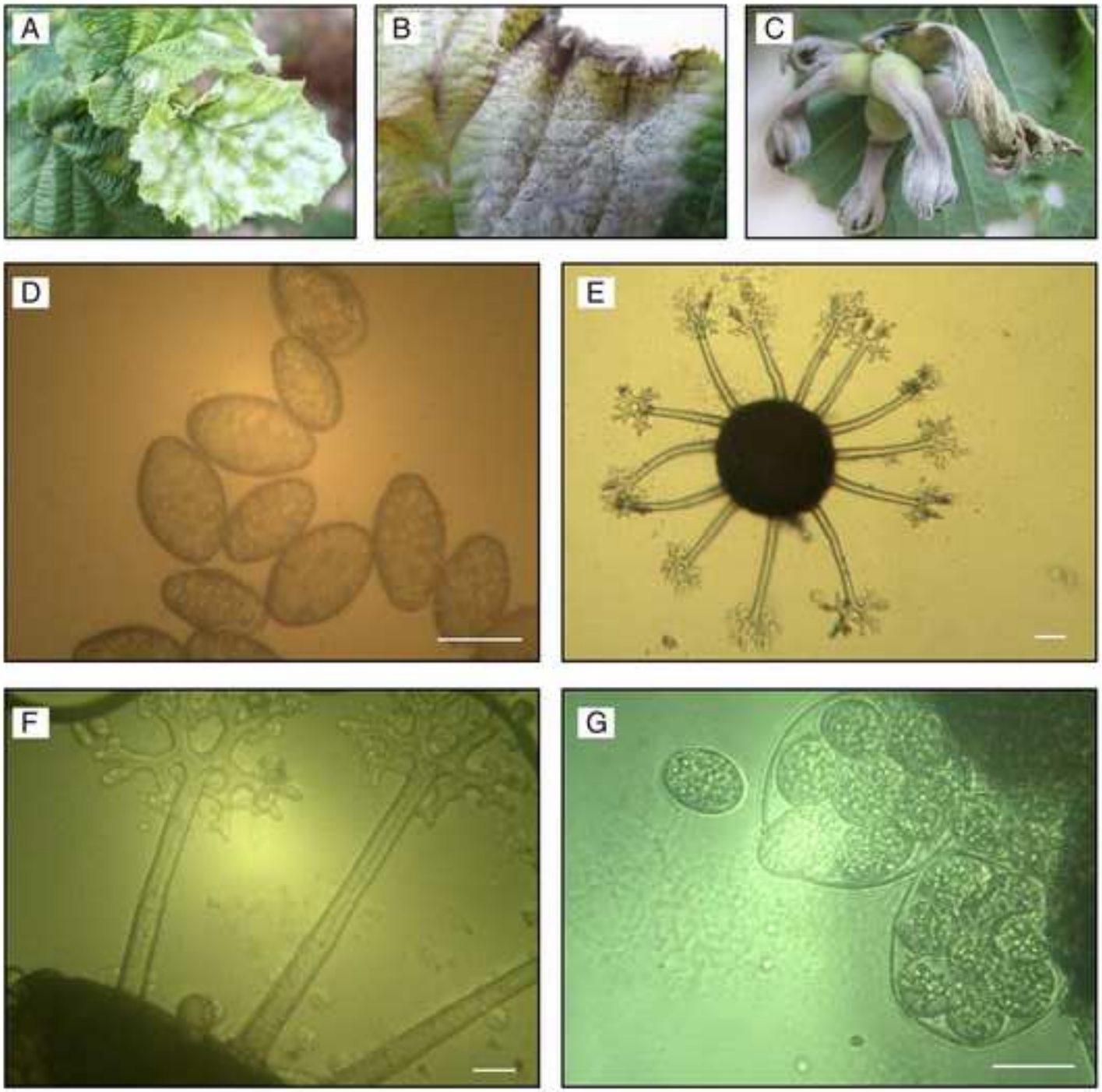

Online Resource 1. Multiple sequence alignment of ITS region of isolate ASezer (KY082910) with other E. corylacearum ITS sequences found in GenBank, listed by their Accession numbers. Only variant bases are shown, with identical bases indicated by dots and gaps by dashes

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KY082910.1     TCA TTA CAG AGT GTG AGG CTC ACT CGT GGC ATC TGC TGC GGG CTG GGC CGA CCC TCC CAC
LC009928.1     --- --- ... ..C ... ... ... ... ... ... ... ... C.. .T. ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     --. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     CCG TGT CGA TTT ATA TCT TGT TGC TTT GGC GGG CCG GGT TGT GTC GTC GCT GCC CGC AAG
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     GAC ATG CGT TGG CCA CCC ACC GGC TTC GGC TGG AGC GCG TCC GCC AAA GAC CTA TAC CAA
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... -.. ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     AAC TCA TGT TGT CTT TGC AGT CTA AGC TTT ATT ATT GAA TTG ATA AAA CTT TCA ACA ACG
LC009928.1     ... ... ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     GAT CTC TTG GCT CTG GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT AAG TAA TGT GAA TTG
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     CAG AAT TTA GTG AAT CAT CGA ATC TTT GAA CGC ACA TTG CGC CCC TTG GTA TTC CGA GGG
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     GCA TGC CTG TTC GAG CGT CAT AAC ACC CCC TCC AGC TGC CTT TGT GTG GTT GCG GTG TTG
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     GGG CAC GTC GCG GTG CGG CGG CCC TTA AAG ACA GTG GCG GTC CCG GCG TGG GCT CTA CGC
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     GTA GTA ACT TGC TTC TCG CGA CAG AGT GAC GAC GGT GGC TTG CCA AAA GCC CTT TTG CTC
LC009928.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
JX880086.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
KR048061.1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

KY082910.1     CAG TCA CAT GGA T-C ACA GGT TGA CCT CGA ATC AGG TAG GAA TAC CCG CTG AAC TTA A--
LC009928.1     ... ... ... ... .-. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .GC
JX880086.1     ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .GC
KR048061.1     ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .GC
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Arzu SEZER[1]

F. Sara DOLAR[2]

S. James LUCAS[3]

Çiğdem KÖSE[1]

Ebru GÜMÜŞ[1]

First Report of an Emerging and Destructive Powdery Mildew Agent *Erysiphe corylacearum* in Hazelnut in Turkey

Authors' address: [1]Ministry of Food Agriculture and Livelihood of Turkey, Hazelnut Research Institute, Teyyaredüzü Mah. Atatürk Bulvarı, 28200 Giresun, Turkey;

[2] Department of Plant Protection, Faculty of Agriculture, University of Ankara, 06110 Ankara, Turkey;

[3]Sabancı University, Nanotechnology Research and Application Center, Orta Mahalle, Üniversite Caddesi No:27, 34956 Tuzla, İstanbul

E-mail address: arsezer@gmail.com

Phone number: +90 538 4951617

Abstract

Hazelnut (*Corylus avellana* L.) is Turkey's most valuable agricultural export, and an essential source of income for many families in the Black Sea Region. In spring 2013, hazelnut leaves, fruit clusters and shoots showing powdery mildew infection symptoms different from those observed previously were discovered in Giresun, Ordu and Trabzon provinces of Turkey. The disease has become epidemic throughout all hazelnut production areas spreading from east to west of the Black Sea Region over the subsequent. This new and highly destructive powdery mildew agent has been identified as *Erysiphe corylacearum* U. Braun & S. Takam. based on its morphological characteristics and DNA sequence of the internal transcribed spacer (ITS) and 28S regions of the ribosomal DNA. Its pathogenicity to this species has been examined in an infection test and proven for the first time. To our knowledge, this is the first report of *E. corylacearum* parasitization of *Corylus avellana* worldwide.

Key words: Hazelnut, powdery mildew, *Erysiphe corylacearum*, Black Sea Region

Introduction

Hazelnut (*Corylus avellana*) is one of the most important tree nut crops in Turkey. As well as having 80% of the world production area, Turkey has the leading position in production and export of hazelnuts (Anonymous, 2017a). Hazelnut production extends along the Black Sea region, from the Georgia border in the east, to Istanbul in the west. Although 33 provinces have hazelnut growing areas, only 16 of them are licensed for growing hazelnut. Almost all production (90 %) is carried out in six provinces: Ordu, Giresun, Trabzon, Samsun, Sakarya and Düzce (Anonymous, 2017b).

For hazelnut, powdery mildew disease is usually the result of *Phyllactinia guttata* (Wallr.: Fr) Lév. infection (Hartney et al., 2005). This is also the case in Turkey, where Bremer (1948) noted the important role played by disease in hazel cultivation, and identified *P. suffulta* (a synonym for *P. guttata*) as the causative agent of powdery mildew. Previous surveys of powdery mildew infection caused by *P. guttata* have found it to be widespread, while in the western part of the Black Sea Region it was found to be the most common disease, with up to 70% infection in hazelnut groves (Yürüt et al., 1994). In spite of powdery mildew being the most widespread disease on Turkish hazelnut production areas, treating it is often regarded as unnecessary because the fungus does not directly affect the nut crop; it causes white powdery growth on the undersides of leaves only late in the season.

However, over the last three years much more serious powdery mildew disease has been observed on cultivated *C. avellana* in the Black Sea Region, with much greater impact on the infected trees. Observations in 2014-2015 showed that this more severe powdery mildew infection is found in a high proportion of hazelnut cultivation areas throughout the eastern Black Sea region and the disease has caused significant damage. The disease was observed in all 16 provinces licensed for hazelnut production in 2016 and its prevalence was 100% in most of them. Here, we report that the causative agent of this disease has been identified as *Erysiphe corylacearum*, a member of the Erysiphaceae family distinct from known powdery mildew agent *Phyllactinia guttata* in hazelnut.

Materials and Methods

Observations of disease symptoms and fungal materials Disease symptoms of powdery mildew were observed in different stages and powdery mildew samples were collected from hazelnut leaves and fruit clusters naturally infected with powdery mildew in hazelnut orchards in 2013-2015 at Black Sea Region (Ordu, Giresun, Trabzon, Samsun, Rize, Artvin, Sakarya and Düzce provinces) of Turkey. Infected fresh materials were used for microscopic observation and DNA extraction.

Morphological observations of the pathogen Sporulating fungal structures were dissected from plant tissues and mounted in distilled water and examined using light microscopy with a camera attachment (Bel-Photonics, STMPRO-T, P.C.R). One hundred of all structures including size of chasmothecia, appendages, asci, ascospores, conidia, peridial cells were measured. Asci numbers / chasmothecia, ascospores numbers /ascus and appendages branching style were also determined and photographed (Bacigalova and Markova 2006). Results were compared with the species descriptions in Braun (1982) and Braun (1987).

DNA extraction and PCR amplification To confirm the morphological identification, sequence analysis of the barcode regions of the fungal ribosomal DNA (rDNA) was carried out. Conidia and chasmothecia of the pathogen were scraped from the surface of infected leaves, and DNA was extracted from 50 µg of fungal tissue using the Nucleospin Plant II kit (Macherey Nagel, Düren, Germany) following the manufacturer's protocol for fungal material. Two different regions of the rDNA, the internal transcribed spacer (ITS) and the variable region of the 28S rRNA gene were amplified by polymerase chain reaction (PCR). In order to ensure specific amplification of fungal DNA rather than any contaminating hazelnut tissue, nested PCR was carried out in both cases using the primers listed in Table 1. PCR amplification was carried out using Maximo Taq polymerase (GeneON, Ludwigshafen am Rhein, Germany) in a Techne TC-Plus thermocycler (Cole-Parmer, Stone, UK) using the following amplification conditions: initial denaturation at 95^{o}C for 5 minutes; 35 cycles of 15s at 95^{o}C, 25s at 52^{o}C, 45s at 72^{o}C; final extension for 7 min at 72^{o}C. PCR products were analyzed by 1.2% agarose gel electrophoresis in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA). For the PMITS1-PMITS2 primer pair, some non-specific amplification was also observed, so the major PCR product was excised from the gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) before proceeding

with the second step of the nested PCR. Final PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Düsseldorf, Germany).

DNA sequencing and data analysis Purified PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for Sanger sequencing. The primers used for the inner amplification step from each nested PCR (Table 1) were also used as sequencing primers for their PCR products. Sequencing chromatograms were visualized, low quality bases trimmed, and forward and reverse reads combined using SeqTrace v0.9.0 (Stucky, 2012). Sequences were compared with known fungal DNA sequences in Genbank using the NCBI Nucleotide BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Zhang et al. 2000). Multiple sequence alignment and phylogenetic tree construction was carried out using MEGA6 software (Tamura et al. 2013).

Pathogenicity test Pathogenicity tests were conducted on vigorous hazelnut suckers having young leaves in an orchard where the disease was not observed. Healthy five leaves of each five suckers were inoculated by gently pressing symptomatic leaves loaded with conidia onto them. Five non-inoculated suckers away from inoculated ones served as a control (Erper et al., 2010). All suckers were covered with transparent plastic bags for two days. The suckers were checked for powdery mildew symptoms occurrence.

Results

The powdery mildew disease caused by the emerging agent develops comparatively early in the spring, with symptoms being observed on leaves, young shoots and immature nut clusters. Initially circular to irregular white patches of mycelium and conidia develop on the both side of leaves. If the colonies first develop on the underside of leaves, it is seen mottled lightening and yellowing of the upper surface of leaves (Fig. 1A). In time the leaves become tarnished, the lesions turn brown in colour and brown-black fungal chasmothecia can be readily observed projecting from them (Fig. 1B) Infected leaves dry out, curl up and fall early. Similar symptoms are observed on the husk of nut clusters (Fig. 1C), and in relatively sensitive varieties they dry out and fall early, leading to crop losses.

Regarding identification, mycelium on leaves were amphigenous, white, persistent, dense, patches. Conidia were produced singly on the conidiophores and measured 32.4 ± 0.4 µm x 20.2 ± 0.3 µm (Fig. 1D). Chasmothecia were scattered to gregarious, 88.0 ± 0.9 µm in diam (Fig. 1E). Peridial cells were polygonal to rounded, 13.3 ±

0.3 µm in diam. Appendages were 6-15 in number, equatorial, stiff, straight, 0.75 to 1.34 times as long as the chasmothecial diam., stalk aseptate, smooth to rough, hyaline, thin in the upper half, thick towards the base, apex 3-5 times closely and regularly branched, tips recurved (Fig. 1F). Each chasmothecium contained 3-5 asci that were mostly sessile, 49.2 ± 0.6 µm x 37.7 ± 0.6 µm, and contained 6-8 ellipsoid-ovoid ascospores, each 19,4 ± 0.3 µm x 12.1 ± 0.2 µm (Fig. 1G) The structures and measurements were in agreement with the descriptions of *Erysiphe corylacearum* U. Braun & S. Takam. (Braun 1982, 1987, 2002).

Morphological identification was confirmed by PCR amplification and sequencing of the internal transcribed spacer (ITS) and 28S rRNA regions of the rDNA gene from a field isolate. DNA isolation, PCR amplification and sequencing were carried out in duplicate using different samples from the same infected tree. The duplicates yielded identical results, giving a 623 bp sequence from the ITS and 843 bp from the 28S rRNA, both of which were deposited in GenBank (Accession Nos. KY082910 & KX279887 respectively). Sequence similarity searches revealed that the closest match, with >99% sequence identity in both regions, was the previously published rDNA sequence from *E. corylacearum* isolate MUMH 0199 (Accession No: LY009928), which was collected from Japanese hazel (*C. sieboldiana*, Takamatsu et al. 2015). In the 28S region, only 2/843 bases differed from the previous sequence. For the ITS region 2 further *E. corylacearum* isolates collected from Asian hazel (*C. heterophylla*, unpublished) were present in GenBank, and multiple alignment of the 4 sequences revealed a total of 7 variant sites (Online Resource 1).

Inoculated hazelnut suckers developed typical powdery mildew symptoms after 10 days, whereas the controls remained symptomless. The fungus present on the inoculated suckers was identical morphologically to that originally observed on diseased plants.

Discussion

For hazelnut, powdery mildew disease is usually the result of *Phyllactinia guttata* (Wallr.: Fr) Lév. infection (Hartney et al., 2005). This is also the case in Turkey, where Bremer (1948) noted the important role played by disease in hazel cultivation, and identified *P. suffulta* (a synonym for *P. guttata*) as the causative agent of powdery mildew. This fungus can infect a diverse range of hard-shelled fruit bearing trees (Pscheidt et al. 2002) in addition to *C. avellana*, and also attacks a very broad range of deciduous trees (Braun 1987). For commercial hazelnut orchards the disease is regarded as not serious enough to warrant control (Hartney et al., 2005, Pscheidt et al. 2002). Regarding the much more serious powdery mildew observed in recent years, the causal agent was

identified here as *Erysiphe corylacearum*, which has been observed to parasitize various Corylus species in the world (Farr and Rossman, 2016). However, this taxon has not been reported to infect *C. avellana* before.

Based on rDNA ITS sequences, new scanning electron microscope (SEM) examinations and other morphological data, Braun & Takamatsu (2000) reassessed the whole complex of powdery mildew genera with Pseudoidium anamorphs and introduced a new circumscription of the genus Erysiphe. Braun (2002) made some additional corrections including *Erysiphe corylacearum* U. Braun & S. Takam. nom. nov. (≡ *Microsphaera hommae* U. Braun, ≡ *Erysiphe hommae* (U. Braun) U. Braun & S. Takam.). Both the morphological and molecular analysis of the powdery mildew pathogen identified in Turkey confirmed that is an example of *E. corylacearum*, although there were a small number of single nucleotide sequence variations compared to previously reported sequences. These differences may indicate that the strain reported here is distinct from those found on Asian and Japanese hazel, and may also be useful as molecular markers for pathogenicity. Future studies will survey samples of the pathogen from across the Black Sea region, to test for diversity within this strain.

Acknowledgments

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Table 1 Primer pairs used in nested PCR amplification of fungal rDNA

Figure 1 Symptoms of powdery mildew on hazelnut leaves (A and B) and fruit cluster (C); conidia (D), bar = 20 μm; chasmothecium (E), bar = 20 μm; appendages (F), bar = 10 μm; asci and ascospores of *Erysiphe corylacearum* (G), bar = 20 μm

Online Resource 1 Multiple sequence alignment of ITS region of isolate ASezer (KY082910) with other *E. corylacearum* ITS sequences found in GenBank, listed by their Accession numbers. Only variant bases are shown, with identical bases indicated by dots and gaps by dashes.

Amplified region	Primer name	Primer sequence	Reference
ITS (Outer)	PMITS1	5’-TCGGACTGGCCCAGGGAGA-3’	Cunnington et al. 2003
	PMITS2	5’-TCACTCGCCGTTACTGAGGT-3’	
ITS (Inner)	ITS5	5’-GGAAGTAAAAGTCGTAACAAGG-3’	White et al. 1990
	ITS4	5’-TCCTCCGCTTATTGATATGC-3’	
28S (Outer)	PM3*	5’-GKGCTYTMCGCGTAGT-3’	Takamatsu & Kano, 2001
	TW14	See below	
28S (Inner)	NL1	5’-AGTAACGGCGAGTGAAGCGG-3’	Mori et al. 2000
	TW14	5’- GCTATCCTGAGGGAAACTTC-3’	

* Degenerate primer. Variable bases are shown using IUPAC ambiguity codes.