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**Molecular Characterization and Pathogenicity of Diaporthe Species Causing Nut Rot of Hazelnut in Italy**

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## 17 **Abstract**

18 Hazelnut (*Corylus avellana*), a nut crop that is rapidly expanding worldwide, is endangered by a  
19 rot. Nut rot results in hazelnut defects. A survey was conducted in north-western Italy during  
20 2020 and 2021 to identify the causal agents of hazelnut rots. Typical symptoms of black rot,  
21 mold, and necrotic spots were observed on hazelnut nuts. The prevalent fungi isolated from  
22 symptomatic hazelnut kernels were *Diaporthe* spp. (38%), *Botryosphaeria dothidea* (26%),  
23 *Diplodia seriata* (14%), and other fungal genera with less frequent occurrences. Among 161  
24 isolated *Diaporthe* spp., 40 were selected for further analysis. Based on morphological  
25 characterization and multi-locus phylogenetic analysis of the ITS, *tefl- $\alpha$* , and *tub2*, seven  
26 *Diaporthe* species were identified as *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D.*  
27 *ravennica*, *D. rudis*, and *D. sojiae*. *D. eres* was the main species isolated from hazelnut rots, in  
28 particular from moldy nuts. Pathogenicity test performed on hazelnut nuts ‘Tonda Gentile del  
29 Piemonte’ using a mycelium plug showed that all the *Diaporthe* isolates were pathogenic on  
30 their original host. To our knowledge, this work is the first report of *D. novem*, *D. oncostoma* and  
31 *D. ravennica* on hazelnut nuts worldwide. *Diaporthe foeniculina*, *D. rudis*, and *D. sojiae* were  
32 reported for the first time as agents of hazelnut nut rot in Italy. Future studies should focus on the  
33 comprehension of epidemiology and climatic conditions favoring the development of *Diaporthe*  
34 spp. on hazelnut. Prevention and control measures should target *D. eres*, representing the main  
35 causal agents responsible for defects and nut rot of hazelnuts in Italy.

36

## 37 **Introduction**

38 Hazelnut (*Corylus avellana* L.) belongs to Betulaceae family, which is native to Europe and  
39 Western Asia, where it is widely distributed (Arciuolo et al. 2020). Hazelnut production

40 increased in the last several years in response to a high demand for its health benefits from fiber  
41 and nutrients (Glei et al. 2018; Nunzio 2019), but also for its massive use in spreadable creams  
42 and other confectionery products (Silvestri et al. 2021). The main producers of hazelnuts are  
43 Turkey, Italy, and the USA. In Italy, hazelnut is the major nut crop, cultivated on 84,440 ha, with  
44 an annual production of about 118,791 t (ISTAT 2022). The cultivation of hazelnut is spread all  
45 over Italy, from north to south, with main production regions as Latium (38%), Campania (37%),  
46 and Piedmont (13%) (Scarpari et al. 2020).

47 Hazelnut production is threatened by various fungal pathogens and disorders which reduce nut  
48 quality and yield by altering its kernel (Arciuolo et al. 2022; Battilani et al. 2018; Teviotdale et  
49 al. 2002) and can release mycotoxins (Spadaro et al. 2020; Valente et al. 2020). Several defects  
50 are reported on hazelnut kernels, such as inner discoloration, necrosis, and presence of  
51 blemishes, which reduce the market quality standards (Arciuolo et al. 2020). Hazelnut rot, called  
52 “avariato” (spoiled) in Italian, affects 3 to 7% of the nuts every year, causing significant  
53 economic losses (Vitale et al. 2020). Different fungal genera were isolated from rotten hazelnuts:  
54 *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Penicillium*  
55 *Pestalotiopsis*, and *Phoma* in the Caucasus region (Battilani et al. 2018); *Alternaria*,  
56 *Colletotrichum*, *Fusarium*, and *Phomopsis* in central Italy (Librandi et al. 2006); *Alternaria*,  
57 *Botryosphaeria*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Didymella*, *Fusarium*, and *Phoma* in  
58 northern and southern Italy (Vitale et al. 2020). Among the fungal pathogens, *Diaporthe* spp.  
59 seem to play a major role in the occurrence of hazelnut defects in the Caucasus region (Arciuolo  
60 et al. 2022; Battilani et al. 2018), but it is unclear which of the above-mentioned fungal genera  
61 play a key role in Italy.

62 The genus *Diaporthe* (anamorph *Phomopsis*) belongs to Diaporthaceae family and was originally  
63 established with *Diaporthe eres* as the typified species isolated from *Ulmus* sp. in Germany  
64 (Nitschke 1870; Senanayake et al. 2017). The members of *Diaporthe* genus represent a  
65 cosmopolitan group of fungi which include plant pathogens, saprobes on decaying tissues and  
66 endophytes widely distributed in tropical, and temperate regions worldwide (Guarnaccia et al.  
67 2018; Marin-Felix et al. 2019; Yang et al. 2020). *Diaporthe* spp. are causal agents of diseases on  
68 a wide range of economically important plant hosts, such as horticultural, forest, ornamentals,  
69 and fruit crops (Bertetti et al. 2018; Dissanayake et al. 2017; Guarnaccia and Crous, 2018; Huang  
70 et al. 2015; Prencipe et al. 2017; Thompson et al. 2011; Udayanga et al. 2014; Yang et al. 2018).  
71 Historically, *Diaporthe* spp. were considered monophyletic group based on unique and typical  
72 *Phomopsis* spp. asexual and sexual morphs (Gomes et al. 2013). However, paraphyletic nature  
73 was revealed by Gao et al. (2017) showing that the genera *Phaeocytostroma*, *Stenocarpella*  
74 (Lamprecht et al. 2011), *Pustulomyces* (Dai et al. 2014), *Ophiodiaporthe* (Fu et al. 2013) and  
75 *Mazzantia* (Wehmeyer 1926) are embedded in *Diaporthe s. lat.* Moreover, Senanayake et al.  
76 (2017) included *Diaporthe*-like clades within the order Diaporthales.

77 Pathogen identification at species level is crucial to understand the biology and epidemiology  
78 and to develop an appropriate disease management (Santos et al. 2017; Yang et al. 2018).  
79 Traditionally, *Diaporthe* species identification was based on culture characteristics, morphology,  
80 and host association (Udayanga et al. 2011; Yang et al. 2020), but morphological identification  
81 was unreliable for species identification because of high similarity of *Diaporthe* spp.  
82 (Dissanayake et al. 2017; Udayanga et al. 2011). Several studies based on the use of multi-locus  
83 phylogenetic analyses solved the boundaries within *Diaporthe* genus (Gomes et al. 2013; Marin-  
84 Felix et al. 2019; Udayanga et al. 2012). Mostly, internal transcribed spacer (ITS), translation

85 elongation factor-1 $\alpha$  (*tef-1 $\alpha$* ), beta tubulin ( *$\beta$ -tubulin*), calmodulin (*CAL*), and histone (*HIS*)  
86 genes are used for molecular characterization of *Diaporthe* spp. (Guarnaccia et al. 2018; Yang et  
87 al. 2020).

88 In Northern Italy, hazelnuts often show nut rot. Several fungi have been associated to nut rot in  
89 Italy (Librandi et al. 2006; Vitale et al. 2020). However, the role of the causal agents, and in  
90 particular of *Diaporthe* spp., has not been clarified. Moreover, it is unclear which species of  
91 *Diaporthe* are involved in the development of hazelnut nut rot. In order to investigate and better  
92 understand the etiology of defected kernels, a monitoring of their possible causal agents was  
93 performed between 2020 and 2021. The aims of the present study were (i) to isolate and identify  
94 the fungal species associated with hazelnut nuts defects in Northern Italy, (ii) to evaluate the  
95 genetic diversity of *Diaporthe* spp. associated with hazelnut nuts defects in Northern Italy, and  
96 (iii) to evaluate the pathogenicity of the species found.

97

## 98 **Materials and methods**

99

### 100 **Sampling and isolation**

101 Field surveys were conducted during November-December each in 2020 and 2021 in nine  
102 orchards located in Piedmont, Northern Italy. A total of 420 nut samples were collected from  
103 defected hazelnuts (moldy, black rotted, and necrotic) at BBCH 89 (nuts separated from the husk  
104 at the basal scar; Paradinas et al. 2022) (**Fig. 1**). Symptomatic kernels cut in half were disinfected  
105 in 1% sodium hypochlorite for 1 min, rinsed in sterile water for 1 min, and dried on sterile filter  
106 paper. Then five small pieces of half cut kernels were placed on potato dextrose agar (PDA,  
107 VWR international, Leuven, Belgium) containing streptomycin (0.025g/l). The PDA plates (9

6

108 cm diameter) were incubated at  $23 \pm 2$  °C with a cycle of 12 h of light and 12 h of darkness for  
109 2-3 days depending on colony growth. Pure cultures were obtained after 10 days by transferring  
110 the mycelium plug from the edge of the colonies and placed in fresh PDA plates. Isolates used in  
111 this study were maintained and kept at -80 °C in the culture collection of the University of Turin,  
112 Torino, Italy.

113

#### 114 **Macro- and micro-morphological analysis**

115 Mycelial plugs (5 mm diameter) were taken from the margins of actively growing colonies on  
116 PDA and transferred onto the center of Petri dishes containing 2% tap water agar supplemented  
117 with sterile pine needles (PNA; Smith et al. 1996) and PDA and incubated at room temperature  
118 ( $22 \pm 2$  °C) under a 12-h near-ultraviolet light/12-h dark cycle to induce sporulation according to  
119 Gomes et al. (2013) and Lombard et al. (2014). Colony characters and pigment production on  
120 PDA and PNA were noted after 7 and 10 days. Colony colors were ranked according to Rayner  
121 (1970). Cultures were observed periodically for the development of ascomata and conidiomata.  
122 Morphological characteristics were examined by mounting fungal structures in clear lactic acid.  
123 Using an Eclipse 55i microscope (Nikon, Tokyo, Japan), 30 measurements at 1000×  
124 magnification were performed per isolate. Initially, identification was performed by colony and  
125 conidial morphology as described by Phillips et al. (2013), and then based on molecular  
126 identification by using internal transcribed spacer sequence amplified with primers ITS 1 and  
127 ITS 4 (White et al. 1990).

128

#### 129 **DNA extraction and PCR amplification**

130 Forty isolates of *Diaporthe* species were selected based on morphological analysis, DNA  
131 sequence, and geographical origin data for further study (**Table S1**).

132 Genomic DNA was extracted from selected isolates of *Diaporthe* spp. using E.Z.N.A. Fungal  
133 DNA mini kit (Omega Bio-tek, Darmstadt, Germany) from 100 mg of mycelium grown on PDA  
134 according to the manufacturer's instructions. Partial regions of three loci were amplified. The  
135 primer sets ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ITS4 (5'-TCCGCTTATTGATATGC-  
136 3') (White et al. 1990) were used to amplify the internal transcribed spacer (ITS) of ribosomal  
137 DNA. Primer pair EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3')/EF1-986R (5'-  
138 TACTTGAAGGAACCTTACC-3') (Carbone and Kohn 1999) were used to amplify partial  
139 translation elongation factor 1- $\alpha$  gene (*tefl- $\alpha$* ), and the beta-tubulin (*tub2*) gene was amplified  
140 using primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC 3') and Bt2b (5'  
141 ACCCTCAGTGTAGTGACCCTTGGC 3'). In case of lack of amplification of beta-tubulin  
142 gene, T1 (5'-AACATGCGTGAGATTGTAAGT-3') and Bt2b (5'-ACCCTCA-  
143 GTGTAGTGACCCTTGGC-3') primers were used (Glass and Donaldson 1995; O'Donnell and  
144 Cigelnik 1997). The PCR amplification mixtures and cycling conditions for all three genes were  
145 performed according to Guarnaccia and Crous (2018). The amplification products were analyzed  
146 on 1% agarose (VWR Life Science AMRESCO® Biochemicals) after staining with GelRed™.  
147 PCR products were purified with the PCR Purification Kit (QIAquick®, Germany) before  
148 sequencing by Macrogen Europe B. V. (Amsterdam, Netherlands). The obtained sequences were  
149 analyzed and using the Geneious v. 11.1.5 program (Auckland, New Zealand).

150

## 151 **Phylogenetic analysis**

152 The sequences obtained from the 40 strains in this study were subjected to a blast search in  
153 NCBI's GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) nucleotide database to determine the  
154 closest species for a taxonomic framework of the studied isolates. The results of Blast analysis  
155 indicated that all the isolates belonged to *Diaporthe* genus. The sequences generated in this study  
156 and reference sequences of *Diaporthe* spp. (Hilário et al. 2021) were initially aligned by using  
157 the MAFFT v. 7 online servers (<http://mafft.cbrc.jp/alignment/server/index.html>) (Katoh and  
158 Standley 2013), and then manually adjusted in MEGA v. 7 (Kumar et al. 2016). To achieve the  
159 sub-genus identification of the *Diaporthe* spp., phylogenetic analysis was performed first  
160 individually for each gene and then as a multilocus analysis of three loci (ITS, *tef-1a* and *tub2*).  
161 The phylogeny was based on Bayesian Inference (BI) and Maximum Parsimony (MP) for the  
162 multi-locus analysis (**Fig. 2**). For BI, the best evolutionary model for each locus was determined  
163 using MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analysis. MrBayes v. 3.2.5  
164 (Ronquist et al. 2012) was used to generate phylogenetic trees under optimal criteria per  
165 partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a  
166 random tree topology. The heating parameter was set at 0.2 and trees were sampled every 1,000  
167 generations. The analysis stopped when the average standard deviation of split frequencies was  
168 below 0.01. The MP analysis was performed using Phylogenetic Analysis Using Parsimony  
169 (PAUP) v. 4.0b10 (Swofford 2003). Phylogenetic relationships were estimated by heuristic  
170 searches with 100 random addition sequences. Tree bisection-reconnection was used, with the  
171 branch swapping option set on 'best trees', with all characters equally weighted and alignment  
172 gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI), and  
173 rescaled consistence index (RC) were calculated for parsimony, and the bootstrap analysis (Hillis

174 and Bull 1993) were based on 1,000 replications. Sequences generated in this study were  
175 deposited in GenBank (**Table S2**).

176

### 177 **Prevalence and distribution of *Diaporthe* spp.**

178 The prevalence of *Diaporthe* spp. in hazelnut nuts collected from different sites in the  
179 investigated area was calculated according to Hilário et al. (2021). The Isolation Rate (RI) was  
180 calculated for each species using the formula:

$$181 \quad \text{RI \%} = [\text{NS} / \text{NI}] \times 100$$

182 where NI was the total number of *Diaporthe* isolates collected during survey, NS was the  
183 number of isolates belonging to same species. Overall isolation rate was also determined by  
184 using the NI value that was equal total number of isolates from hazelnut nuts.

185

### 186 **Pathogenicity trial**

187 Pathogenicity of 40 *Diaporthe* strains was evaluated on detached ripening hazelnut nuts ‘Tonda  
188 Gentile del Piemonte’ (BBCH: 85; over 50% of the shells changed color) (Table S3). Nuts were  
189 surface disinfected with 1% NaClO and a piece of shell (5 mm diameter) was removed with a  
190 sterile cork borer. A mycelium plug of 5 mm in diameter was taken from seven days old PDA  
191 colony and placed with the mycelium in contact with the nuts inside each well in the shell. Each  
192 inoculation point of nut was wrapped with Parafilm to avoid dehydration. Three replicates per  
193 isolate and three nuts per replicate were tested. Nine control nuts were treated with a sterilized  
194 PDA plugs as described above and served as negative control. Inoculated nuts were placed in  
195 plastic trays and covered with plastic foil and inoculated at 22±2 °C for 30 days in a chamber,  
196 with a 12 h light/12 h dark period each day. At the end of the incubation period disease severity

197 was calculated by using a disease rating scale from 0 to 4 where 0= no visible symptoms, 1=  
198 <25% development of pycnidia, 2= 25-50% development of pycnidia, 3=50-75% development of  
199 pycnidia 4= $\geq$ 75% development of pycnidia (**Fig. 3**).

200 Analysis of variance (ANOVA) was utilized for statistical analysis. The mean values were  
201 separated by Tukey HSD ( $P < 0.05$ ) by using SPSS software (IBM SPSS Statistics v. 28.0.1.0).

202

## 203 **Results**

### 204 **Fungal isolation and identification**

205 On hazelnut nuts with defects, three types of symptoms were observed: black rot, moldy, and  
206 necrotic spots. Kernels were covered by white-grey mold and a few of them were totally rotten,  
207 wet, and almost black (**Fig. 1**). Fungi were identified based on their symptoms type, morphology  
208 (conidial morphology, colony shape, and color), and, when necessary, by using ITS sequencing.

209 The prevalent fungi isolated from symptomatic hazelnut kernels were *Diaporthe* spp. (38%),  
210 *Botryosphaeria dothidea* (26%), and *Diplodia seriata* (14%). Moreover, *Alternaria* spp.,  
211 *Aspergillus* spp., *Fusarium* spp., Mucorales, *Neofusicoccum* spp., *Penicillium* spp., and  
212 *Trichothecium roseum* were isolated from nuts but with incidence lower than 6%. *Diaporthe* spp.  
213 were isolated from moldy nuts (68%), followed by necrotic nuts (17%) and black rotted nuts  
214 (15%). Members of Botryosphaeriaceae and *Alternaria* spp. were isolated only from black rotted  
215 and necrotic nuts. Whilst *Fusarium* spp. and *Trichothecium roseum* were isolated only from  
216 moldy nuts. Other fungal pathogens were isolated from mixed nuts (moldy, necrotic or black  
217 rotted).

218 *Diaporthe* spp. were isolated from all locations with different frequency: Lu and Cuccaro (30%),  
219 Murazzano (82%), Marsaglia (64%), Rodello (47%), Belvedere Langhe (32%), Cravanzana  
220 (36%), Cortemilia (50%), Borgo D'Ale (8%), and Cavaglià (42%) (**Table 1**).

221

## 222 **Phylogenetic analysis**

223 To further explore the diversity of the *Diaporthe* spp. isolates and to assign the species, three  
224 genes were sequenced: ITS, *tub2*, and *tef-1 $\alpha$* . A combined alignment of the three loci was  
225 analyzed. The combined alignment consisted of 111 sequences including the outgroup  
226 *Diaporthella corylina* (CBS 121124). The final dataset comprised of 1,362 nucleotides (ITS: 1-  
227 473, *tub2*: 480-876, *tef-1 $\alpha$* : 883-1,362). A total of 559 nucleotides were parsimony-informative,  
228 194 were variable and parsimony-uninformative, and 597 were constant. A maximum of 1,000  
229 equally MP trees were saved (Tree length = 3049, CI = 0.471, RI = 0.872, and RC = 0.410).  
230 Bootstrap support values from the MP analysis are included on the Bayesian tree in **Fig. 2**. For  
231 the BI, MrModeltest suggested that all partitions should be analyzed with Dirichlet state  
232 frequency distributions. The following models were recommended by MrModeltest and used:  
233 GTR+I+G for ITS and *tef-1 $\alpha$* , and HKY+G for *tub2*. In the BI, the ITS partition had 179 unique  
234 site patterns, the *tef-1 $\alpha$*  partition had 350 unique site patterns, the *tub2* partition had 259 unique  
235 site patterns and the analysis ran for 3,495,000 generations, resulting in 3,496 trees of which  
236 2,622 trees were samples to calculate the posterior probabilities. Multi-locus phylogenetic  
237 analysis of 40 isolates showed that 28 isolates clustered with *D. eres*, 3 isolates clustered with *D.*  
238 *novem*, 3 with *D. foeniculina*, and 3 with *D. rudis*. Hm-20a-2, HBr-9b-1 and HM-30-2 formed  
239 distinct lineage with *D. oncostoma*, *D. ravennica*, and *D. sojajae*, respectively.

240

## 241 **Morphology**

242 Morphological observations from edges of the Petri dishes, supported by phylogenetic inference,  
243 were used to describe the seven species of *Diaporthe* spp. (**Fig. 4 A-I**).

244 Colonies of *Diaporthe sojae* growing on PDA reached 90 mm within 10 days at 25-26 °C.  
245 Colonies were white, with fluffy aerial mycelium. Reverse colonies were creamy and later  
246 developed a yellowish pigmentation in center of the Petri dish. Alpha-conidia were hyaline,  
247 aseptate, abundant, smooth, ellipsoidal and biguttulate with dimensions  $5.8-7.3 \times 2-3.2 \mu\text{m}$  mean  $\pm$   
248 SD =  $6.67 \pm 0.78 \times 2.57 \pm 0.60$  (**Fig. S1 A-B; Fig. S2 A**).

249 Ten days colonies of *Diaporthe foeniculina* growing on PDA reached 90 mm at 25-26 °C.  
250 Colonies were white with fluffy aerial mycelium. Reverse colonies had green to yellowish  
251 pigmentation. Alpha conidia were hyaline, aseptate, smooth, ellipsoidal or fusiform, with one or  
252 many guttules with dimensions  $7.6-9.5 \times 2.5-2.9 \mu\text{m}$  mean  $\pm$  SD =  $8.6 \pm 0.96 \times 2.7 \pm 0.21$  (**Fig. S1**  
253 **C,D; Fig. S2 B**).

254 *Diaporthe novem* had ten-day colonies on PDA reaching 90 mm at 25-26 °C. Colonies were flat,  
255 fluffy, and white to greyish colonies in center with white aerial mycelium at the margins of the  
256 Petri dish. Colony reverse had black circles and white margins. Alpha-conidia were hyaline,  
257 unicellular, oval to cylindrical, biguttulate with obtuse ends, with dimensions of  $6.9-9.1 \times 2.5-2.9$   
258  $\mu\text{m}$ , mean  $\pm$  SD =  $8.1 \pm 1.11 \times 2.73 \pm 0.21 \mu\text{m}$  (**Fig. S1 E-F; Fig. S2 C**).

259 Ten days colonies of *Diaporthe ravennica* growing on PDA reached 90 mm at 25-26 °C.  
260 Colonies were white, spreading to the edge in wavy appearance, with pycnidia production in  
261 middle of plate, medium flat or dense. Reverse colony was creamy, radiating white outwardly  
262 with black dots in middle. Alpha-conidia were smooth, hyaline, aseptate, multi-guttulate, ovate

263 to ellipsoidal with dimensions 8.3-11.9x2.5-4.3  $\mu\text{m}$  mean  $\pm$  SD =10.1 $\pm$ 1.80x3.30 $\pm$ 0.92 (**Fig. S1**  
264 **G-H; Fig. S2 D**).

265 Colonies of *Diaporthe rudis* growing on PDA reached 85 mm within 4 weeks at 25-26 °C.  
266 Colonies were white and turn brown gradually, fluffy, and flat, radiating outwardly to the edge,  
267 with brown mycelium. Colony reverse was greyish with brown halos. Alpha conidia were  
268 ellipsoidal, hyaline, biguttulate and smooth. Dimensions of conidia were 7.5-8.5x2.4-2.7  $\mu\text{m}$   
269 mean  $\pm$  SD =7.97 $\pm$ 0.50x2.57 $\pm$ 0.15  $\mu\text{m}$  (**Fig. S1 I-J; Fig. S2 E**).

270 Colonies of *Diaporthe eres* were white greyish, fluffy aerial mycelium with abundant pycnidia at  
271 maturity. Colony reverse had dark pigmentation in center and whitish from edges of the Petri  
272 dish. Colonies growing on PDA reached 90 mm at 25-26 °C after 10 days. Alpha conidia were  
273 ovate to ellipsoidal, aseptate, smooth, hyaline, biguttulate, and base subtruncate with dimensions  
274 of 8.5-11.1x 3.5-4.5  $\mu\text{m}$ , mean  $\pm$  SD =9.7 $\pm$ 1.31x3.67 $\pm$ 0.76 (**Fig. S1 K-L; Fig. S2 F**).

275 Colonies of *Diaporthe oncostoma* were smooth and flat with no exudate. Dense fluffy mycelium  
276 was observed. Colony reverse had yellowish green and periphery umber. Colonies growing on  
277 PDA reached 25 mm at 25-26 °C after 10 days. Alpha conidia were hyaline, septate, and bent  
278 cylindrical with dimensions of 11-14.5x 3.2-4.9  $\mu\text{m}$ , mean  $\pm$  SD = 12.83 $\pm$ 1.76x4.20 $\pm$ 0.89 (**Fig.**  
279 **S1 M-N; Fig. S2 G**).

280

### 281 **Prevalence and distribution of *Diaporthe* spp.**

282 *Diaporthe* spp. were isolated from all the sites surveyed (Lu and Cuccaro, Murazzano,  
283 Marsaglia, Rodello, Belvedere Langhe, Cravanzana, Cortemilia, Borgo D'Ale, and Cavaglià)  
284 showing symptoms of black rot, moldy, and necrosis. In particular, *Diaporthe* spp. were  
285 predominantly isolated from moldy nuts. Based on morphology, among 161 *Diaporthe* spp.

286 isolates, *D. eres* was the dominant species, representing 92.5 % of the isolates collected. Three  
287 strains per species of *D. rudis*, *D. novem*, and *D. foeniculina* were isolated. Only one isolate was  
288 found per each of the species *Diaporthe oncostoma*, *D. sojae*, and *D. ravennica*.

289 *Diaporthe eres* was predominantly isolated in all the geographical areas: it was the only species  
290 isolated in Belvedere Langhe, Cravanzana, Cortemilia, and Borgo D'Ale. Moreover, it  
291 represented the most isolated species in the other geographical areas: 94 %, 88 %, 93 %, 88 %,  
292 and 86% of the collected isolates were *D. eres* in Lu and Cuccaro, Murazzano, Marsaglia,  
293 Rodello, and Cavaglià, respectively. Whilst *D. rudis* and *D. novem* were recovered only from  
294 three and two towns, respectively, with an abundance lower than 15 % (**Fig. 5**).

295

### 296 **Pathogenicity test**

297 After fifteen days, all the *Diaporthe* isolates developed lesions on the surface of inoculated  
298 kernels. Moreover, the inoculated kernels showed abundant development of pycnidia with  
299 different disease severity (disease rating scale 0-4) on the whole nut as shown in **Fig. 3 and**  
300 **Table S3**. In few cases internal black discoloration was also observed on nuts along with  
301 necrotic spots of variable sizes. All the *Diaporthe* isolates were successfully reisolated from all  
302 the inoculated nuts, fulfilling Koch's postulates. Control hazelnut kernels showed no internal or  
303 external symptoms.

304

### 305 **Discussion**

306 Hazelnut kernel defects are a serious threat for hazelnut yield, quality, and market value. The  
307 present study aimed to investigate the causal agents and the diversity of fungal species associated  
308 with rotten hazelnut nuts in Italy. During the survey performed from 2020 to 2021 in northern

309 Italy, we isolated and identified *Alternaria* spp., *Aspergillus*, *B. dothidea*, *Diaporthe* spp., *D.*  
310 *seriata*, *Fusarium* spp., Mucorales, *Neofusicoccum* spp., *Penicillium* spp., and *T. roseum* based  
311 on morphological and ITS analysis. Distinct taxonomic groups were isolated from different  
312 categories of symptoms. Members of Botryosphaeriaceae and *Alternaria* spp. were isolated only  
313 from black rotted and necrotic nuts. Whilst *Fusarium* spp. and *Trichothecium roseum* were  
314 isolated only from moldy nuts. Previously, *N. parvum* (Waqas et al. 2022), *Alternaria* spp.  
315 (Battilani et al. 2018; Belisario et al. 2004), *B. dothidea* and *D. seriata* (Luna et al. 2022) were  
316 isolated from rotten hazelnuts or other nut crops.

317 *Diaporthe* spp. were the most dominant fungi isolated from defected hazelnut nuts in northern  
318 Italy. Most *Diaporthe* spp. were isolated from moldy nuts (68%), but also from necrotic and  
319 black rotted nuts. The prevalence of *Diaporthe* spp. associated with nut rot is closely correlated  
320 to the sampling area and to the type of nut symptoms. The highest diversity of *Diaporthe* spp.  
321 was observed in Murazzano, where 51% nuts were moldy which is in agreement with previous  
322 studies on hazelnuts from Georgia and Turkey (Battilani et al. 2018; Arciuolo et al. 2020).  
323 *Diaporthe* spp. have a wide host range (Gomes et al. 2013; Lombard et al. 2014; Udayanga et al.  
324 2015; Yang et al. 2021) and were previously reported as causal agents of diseases of hazelnut  
325 nuts (Arciuolo et al. 2020; Bai et al. 2022; Battilani et al. 2018; Gao et al. 2021; Guerrero et al.  
326 2020) and other nut crops (Eichmeier et al. 2020; Lawrence et al. 2015; León et al. 2020; Yang  
327 et al. 2018). Previously, *Diaporthe* spp. were isolated from hazelnut nuts and identified at genus  
328 level in Italy (Vitale et al. 2020).

329 ITS sequence is commonly used for species identification of Diaporthaceae and  
330 Botryosphaeriaceae (Hyde et al. 2014; Marin-Felix et al. 2019). However, ITS sequence is not  
331 informative to distinguish the *Diaporthe* species because of greater intraspecific variation in ITS

332 locus as compared to interspecific variation (Chaisiri et al. 2021; Santos et al. 2010). Therefore, a  
333 multi-locus phylogenetic analyses approach is used for accurate identification and resolution of  
334 *Diaporthe* species (Guarnaccia et al. 2020; Lesuthu et al. 2019; Santos et al. 2017; Zapata et al.  
335 2020). DNA sequence data combined with morphology has been extensively used to establish  
336 the species boundaries in *Diaporthe* genus (Gao et al. 2017; Hilario et al. 2021).

337 Seven *Diaporthe* spp. were identified in this study based on morphological features and three  
338 genomic regions ITS, *tef-1 $\alpha$*  and *tub2*. The closest taxa of the seven *Diaporthe* spp. recovered in  
339 this study were included in the analysis, based on BLASTn search in NCBI's GenBank  
340 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) nucleotide database. The final multilocus phylogenetic  
341 tree differentiated *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and  
342 *D. sojæ* on hazelnut nuts.

343 *D. eres* was the most frequently isolated species associated with defected hazelnut nuts from all  
344 the areas of northern Italy. *D. eres* is an important plant pathogen which can infect various hosts  
345 (Bai et al. 2022) and has been considered the main causal agent of hazelnut nuts defects in the  
346 Caucasus region (Arciuolo et al. 2020; Battilani et al. 2018). Although *D. eres* was reported on  
347 hazelnut nuts in Italy and Turkey by Arciuolo et al. (2020), most isolates could not be classified  
348 at species level due to the poorly supported or non-monophyletic clade. Moreover, pathogenicity  
349 of *D. eres* isolates was unknown on hazelnut nuts. However, our analysis based on three loci,  
350 combined with morphological observations, clearly identified *D. eres* at species level.  
351 Previously, *D. eres* was associated with hazelnut canker in China and the USA (Bai et al., 2022;  
352 Gao et al., 2021; Wiman et al. 2019), and on other hosts (Guarnaccia et al. 2018; Hilário et al.  
353 2021; Lombard et al. 2014; Udayanga et al. 2014; Wang et al. 2021).

354 Previous studies showed that *D. foeniculina* and *D. rudis* could be pathogenic or saprophytic on  
355 different host plants (Gajanayake et al. 2020; Marin-Felix et al. 2019; Udayanga et al. 2014).  
356 Recently, Guerrero et al. (2020) described *D. foeniculina* associated with black tip and necrotic  
357 spots on hazelnut kernel in Chile and stem and shoot cankers on sweet chestnuts in Italy (Annesi  
358 et al. 2016). Moreover, kernel mold of hazelnut was caused also by *D. rudis* in the USA  
359 (Pscheidt et al. 2019) and on other hosts in Italy (Dissanayake et al. 2017; Guarnaccia et al.  
360 2020). Similarly, we detected these species associated with nut rot of hazelnut nuts in Italy.  
361 Moreover, *D. sojae* was isolated and identified for the first time from infected nuts of hazelnut in  
362 Italy, where it was already reported on hazelnut in Turkey (Arciuolo et al. 2020) and on *Glycine*  
363 *soja* in Italy (Gomes et al. 2013). There are no previous records of *D. novem*, *D. ravennica*, and  
364 *D. oncostoma* on hazelnut nuts. So, this study suggests that hazelnut could be a host for *D.*  
365 *novem*, *D. ravennica*, and *D. oncostoma*. Previously, *D. novem* was reported on *Citrus* spp. in  
366 Italy (Guarnaccia and Crous 2017) and on almond in the USA (Lawrence et al. 2015), whilst *D.*  
367 *ravennica* and *D. oncostoma* were reported on *Salvia* sp. and *Tamarix* sp. in Italy (Dissanayake  
368 et al. 2017; Thambugala et al. 2017) and *Robinia pseudoacacia* by Gomes et al. (2013)  
369 respectively.

370 In our phylogeny, the three loci used in this study were unable, when considered singularly, to  
371 discriminate *D. revennica* from *D. foeniculina*, and *D. baccae*. Separation of *D. ravennica* from  
372 *D. foeniculina*, and *D. baccae* was possible only with a combined analysis of ITS, *tef-1 $\alpha$* , and  
373 *tub2* regions as reported in previous studies (Aiello et al. 2022; Gajanayake et al. 2020;  
374 Phukhamsakda et al. 2020). *D. sojae* strain also formed a well-supported clade with CBS 139282  
375 (Udayanga et al. 2015). Our results of phylogeny showed a large diversity of *Diaporthe* spp.  
376 comprising several clades and species, associated with nut rot of hazelnut nuts in Italy.

377 The pathogenicity tests showed that all the studied isolates were pathogenic on hazelnut nuts,  
378 resulting in different disease severity. A total of 40 strains of *Diaporthe*, belonging to *D. eres*, *D.*  
379 *foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojiae* were able to cause  
380 nut rot of hazelnut. *D. eres* was the most virulent species as compared to the other species of  
381 *Diaporthe* which is in agreement with previous studies (Arciuolo et al. 2020; Bai et al., 2022;  
382 Battilani et al. 2018; Gao et al., 2021). Whereas *D. oncostoma* was comparatively less  
383 pathogenic (smaller lesions), suggesting that *D. oncostoma* is probably a weak pathogen on  
384 hazelnut nuts. The other species were moderately pathogenic on their original host.

385 In conclusion, this study elucidates the species of *Diaporthe* associated with defected hazelnut  
386 nuts in northern Italy, by using morphology analysis, molecular data, and pathogenicity. This is  
387 the first report worldwide about nut rot of hazelnut caused by *D. novem*, *D. oncostoma*, and *D.*  
388 *ravennica*, and the first report of *D. foeniculina*, *D. rudis*, and *D. sojiae* as agents of nut rot of  
389 hazelnut in Italy.

390 In this study, we also observed the co-occurrence of *Diaporthe* spp. in the same nuts with  
391 members of Botryosphaeriaceae, as previously reported (Elfar et al. 2013; Guarnaccia et al.  
392 2016; Moral et al. 2017). Such co-existence is not new, as it has been previously described on  
393 branch cankers and stem-end rot of different hosts in Italy and on hazelnut nuts in Caucasus  
394 region (Battilani et al. 2018; Guarnaccia et al. 2016; 2020). Furthermore, *Diaporthe* spp. are  
395 cosmopolitan fungi and can infect a range of cultivated crops and natural ecosystem as a plant  
396 pathogens or saprophytes. Taking all of this into consideration, it deserves further investigation  
397 that *Diaporthe* spp. with other members of Botryosphaeriaceae, may contribute to cause fungal  
398 trunk disease (FTD) of hazelnuts in Italy, that could be a threat to European hazelnut production.

399 Future studies should also focus on elucidating the epidemiology of this disease and on the  
400 environmental conditions favoring the development of *Diaporthe* spp. on hazelnut. Prevention  
401 and control measures should target *D. eres*, which showed to be the main responsible species of  
402 defects on hazelnuts in Italy.

403

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## Tables

**Table 1:** Overall incidence of all *Diaporthe* spp. on hazelnut nuts (*Corylus avellana*) variety and date of cultivation from different towns in Piedmont, Italy

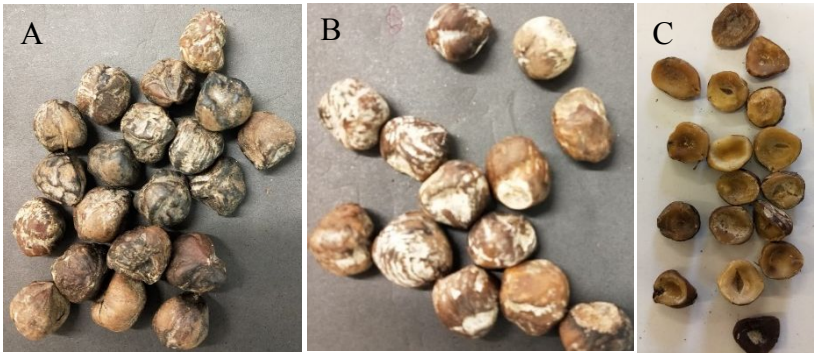
<b>Sr. No.</b>	<b>Towns/Province <sup>1</sup></b>	<b>Variety <sup>2</sup></b>	<b>Date of orchard planting <sup>3</sup></b>	<b><i>Diaporthe</i> incidence (%)</b>
1	Lu and Cuccaro (AL)	TGP	NA	30.43
2	Murazzano (CN)	TGP	2010	82
3	Marsaglia (CN)	TGP	1995	63.6
4	Rodello (CN)	TGP	1990	47
5	Cravanzana (CN)	TGP	1995	36.3
6	Cortemilia (CN)	TGP	1980	50
7	Borgo D'Ale (VC)	TGP	2011	8.3
8	Cavaglia (BI)	TGP	2013	42.4
9	Belvedere Langhe (CN)	TGP	1975	32

<sup>1</sup> Provinces are administrative areas in Italy: AL (Alessandria); CN (Cuneo); BI (Biella); VC (Vercelli).

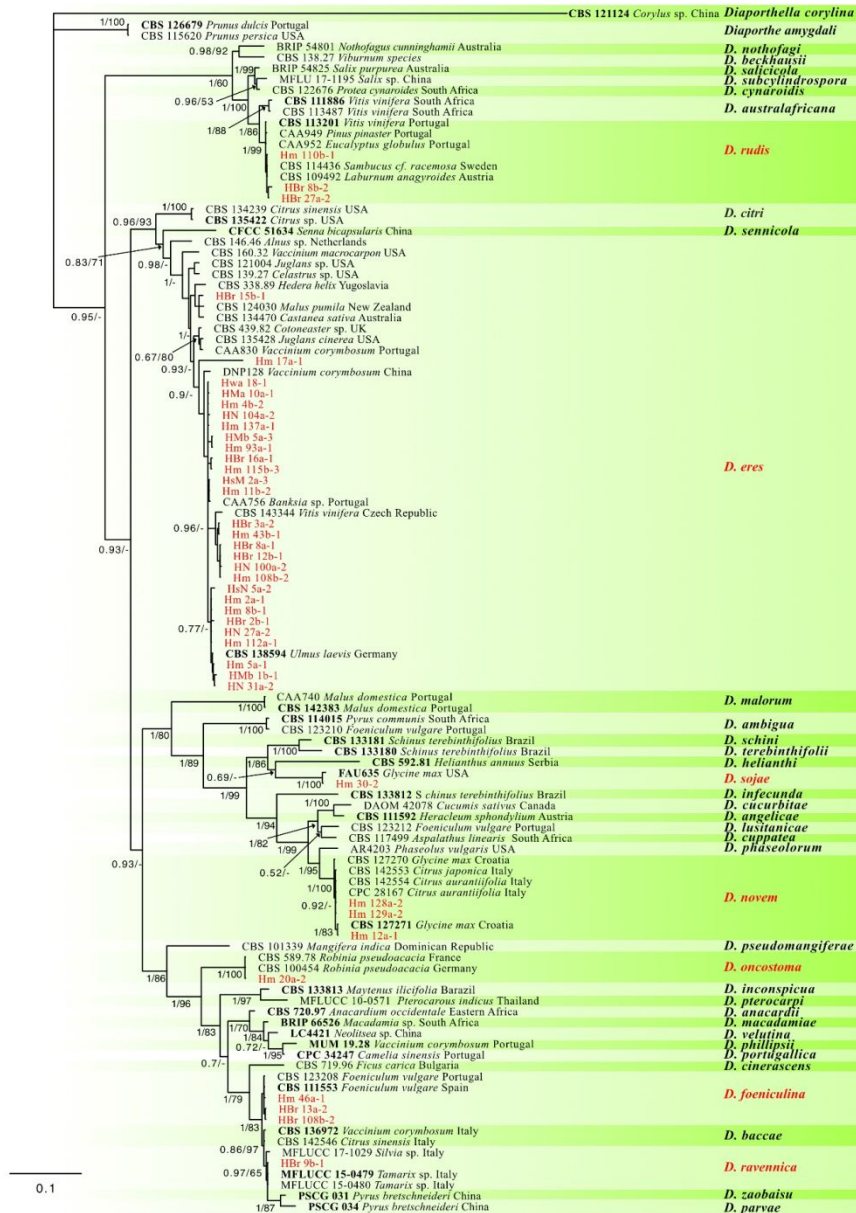
<sup>2</sup> TGP: 'Tonda Gentile del Piemonte'

<sup>3</sup> NA: not available

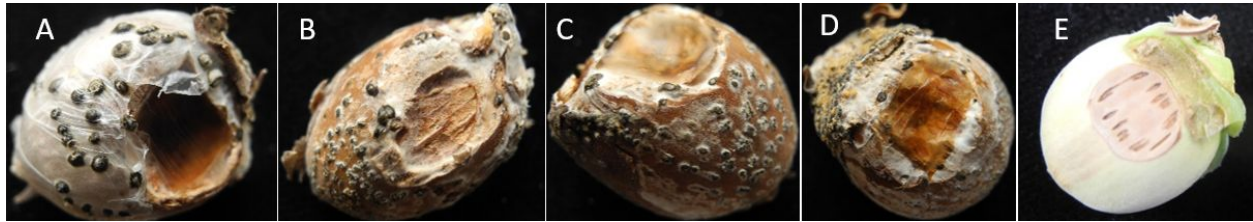
**Figures**



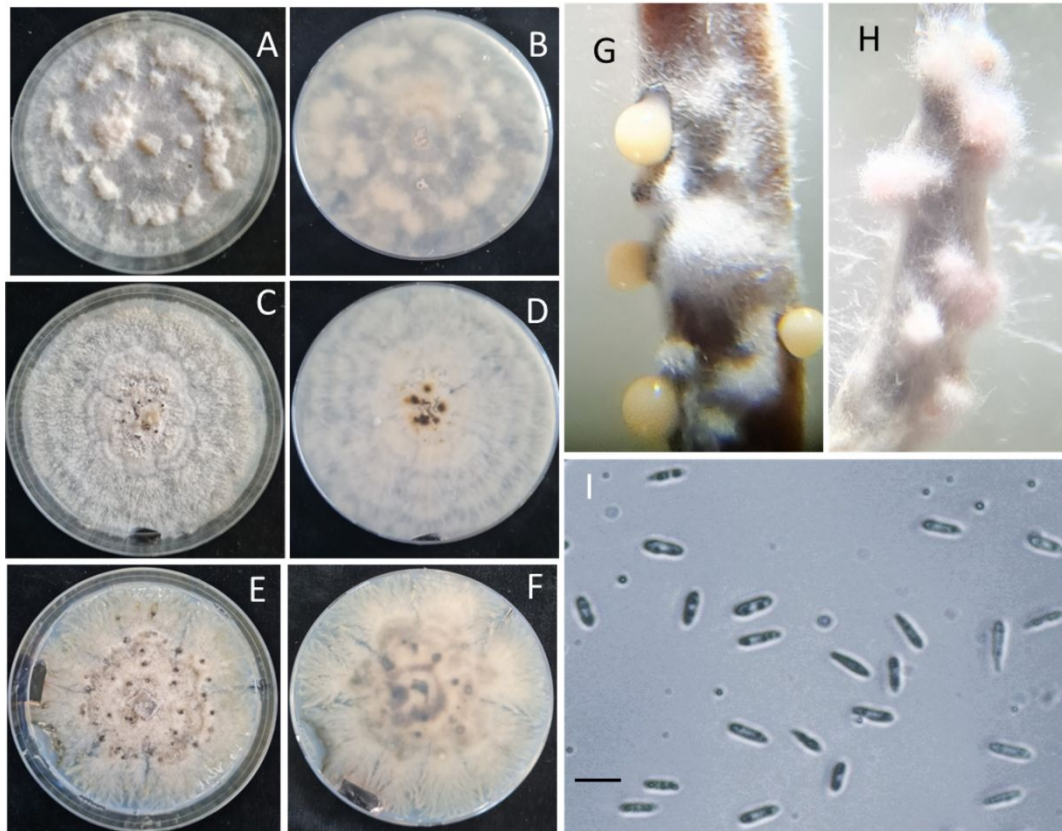
**Fig. 1.** Symptoms observed on hazelnut (*Corylus avellana*) described as (A) black rot (B) moldy (C) necrotic.



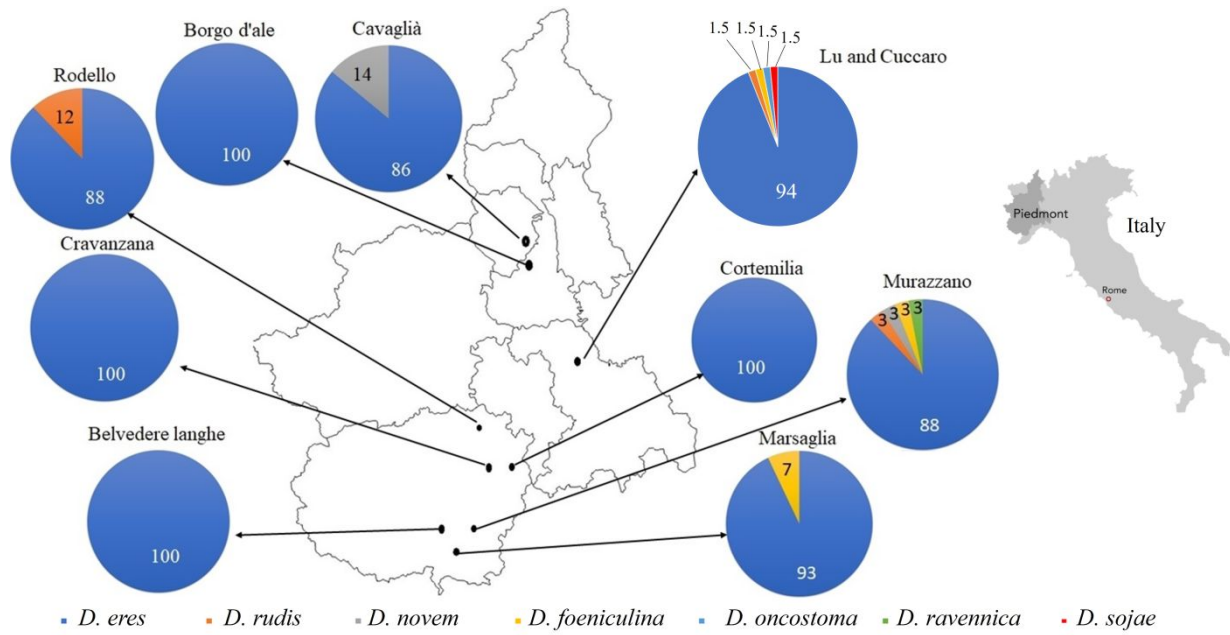
**Fig. 2.** Consensus phylogram of 2,622 trees resulting from a Bayesian analysis of the combined ITS, *tef-1α*, and *tub2* sequence alignments of the *Diaporthella* species. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. Host and country of origin are listed next to the strain numbers. Ex-type isolates are indicated in bold. The isolates obtained in this study are in red. The tree was rooted with *Diaporthella corylina* (CBS 121124).



**Fig. 3.** Symptomatology induced on hazelnut nuts ‘Tonda Gentile del Piemonte’ inoculated with *Diaporthe* spp. and stored at  $22\pm 2$  °C for 30 days in a chamber with a 12 h light/12 h dark. Symptoms of nut rot were evaluated by the following scale: 0: no visible symptoms (**E**); 1: <25% development of pycnidia (**D**); 2: 25-50% development of pycnidia (**C**); 3: 50-75% development of pycnidia (**B**); 4:  $\geq 75\%$  development of pycnidia (**A**).



**Fig. 4** Morphological features of *Diaporthe* spp. obtained from hazelnut nuts. **(A-B)** *D. sojae*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C **(C-D)** *D. ravennica*. Front and reverse side of colony grown on PDA **(E-F)** *D. eres*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C. **(G)** Conidiomata of *D. eres* on sterilized pine needle on WA. **(H)** Conidiomata of *D. sojae* on sterilized pine needle on WA **(I)** Conidia of *D. eres* (dimensions: 8.5-11.1x 3.5-4.5  $\mu\text{m}$ ; scale bar: 10  $\mu\text{m}$ ).



**Fig. 5.** Incidence (%) and distribution of *Diaporthe* species from hazelnut nuts (*Corylus avellana*) according to their sampling locations of Piedmont, Italy.

## e-Xtra (Supplementary) Files

Table S1.

Isolates of *Diaporthe* spp. across nine regions of Piedmont, Italy, obtained from hazelnut (*Corylus avellana*) during 2020-2021 and their GenBank accession numbers.

Sr. no.	Isolate code	Fungal species	Isolation year	Towns/Provinces <sup>1</sup>	Altitude	GenBank Accession <sup>2</sup>		
						ITS	<i>tef1-α</i>	<i>tub2</i>
1	Hwa-18-1	<i>Diaporthe eres</i>	2020	Lu and Cuccaro (AL)	307 m	OM331706	ON933990	OP186258
2	HsN5a-2	<i>D. eres</i>	2020	Lu and Cuccaro (AL)	307 m	OM331707	ON933991	OP186259
3	HsM2a-3	<i>D. eres</i>	2020	Lu and Cuccaro (AL)	307 m	OM331708	ON933992	OP186260
4	HMb-1b-1	<i>D. eres</i>	2020	Lu and Cuccaro (AL)	307 m	OM331709	ON933993	-
5	HMb-5a-3	<i>D. eres</i>	2020	Lu and Cuccaro (AL)	307 m	OM331710	ON933994	OP186261
6	Hma-10a-1	<i>D. eres</i>	2020	Lu and Cuccaro (AL)	307 m	OM331711	ON933995	OP186262
7	HM-2a-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331712	ON933996	OP186263
8	HM-4b-2	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331713	ON933997	OP186264
9	HM-5a-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331714	ON933998	OP186265
10	HM-8b-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331715	ON933999	OP186266
11	HM-11b-2	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331716	ON934000	OP186267
12	Hm-12a-1	<i>D. novem</i>	2020	Murazzano (CN)	739 m	OM331738	ON933987	OP156876
13	HM-17a-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331717	ON934001	OP186268
14	Hm-20a-2	<i>D. oncostoma</i>	2020	Murazzano (CN)	739 m	ON911324	ON934002	OP186269
15	HBR-2b-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331718	OP288105	OP186270
16	HBR-3a-2	<i>D. eres</i>	2020	Murazzano (CN)	739 m	ON911327	OP296410	OP186271
17	HBR-8a-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331719	ON934003	OP186272
18	HBR-8b-2	<i>D. rudis</i>	2020	Murazzano (CN)	739 m	OM331733	ON934004	OP186273
19	HBR-9b-1	<i>D. ravennica</i>	2020	Murazzano (CN)	739 m	ON911325	ON934005	OP186274
20	HBR-12b-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331720	ON934006	OP186275
21	HBR-13a-2	<i>D. foeniculina</i>	2020	Murazzano (CN)	739 m	ON911322	ON934007	OP186276

22	HBR-15b-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331721	ON934008	OP186277
23	HBR-16a-1	<i>D. eres</i>	2020	Murazzano (CN)	739 m	OM331722	ON934009	OP186278
24	HN-27a-2	<i>D. eres</i>	2021	Lu and Cuccaro (AL)	307 m	OM331723	ON934010	OP186279
25	HBr-27a-2	<i>D. rudis</i>	2021	Lu and Cuccaro (AL)	307 m	OM331734	ON934011	OP186280
26	Hm-30-2	<i>D. sojæ</i>	2021	Lu and Cuccaro (AL)	307 m	ON911326	ON934012	OP186281
27	HN-31a-2	<i>D. eres</i>	2021	Lu and Cuccaro (AL)	307 m	OM331724	ON934013	OP186282
28	HM-43b-1	<i>D. eres</i>	2021	Lu and Cuccaro (AL)	307 m	OM331725	ON934014	OP186283
29	Hm-46a-1	<i>D. foeniculina</i>	2021	Lu and Cuccaro (AL)	307 m	ON911323	ON934015	-
30	HM-93a-1	<i>D. eres</i>	2021	Cortemilia (CN)	247 m	OM331726	ON934016	OP186284
31	HN-100a-2	<i>D. eres</i>	2021	Cravanzana (CN)	580 m	OM331727	ON934017	OP186285
32	HM-108b-2	<i>D. eres</i>	2021	Marsaglia (CN)	550 m	OM331728	ON934018	OP186286
33	HN-104a-2	<i>D. eres</i>	2021	Belvedere Langhe (CN)	679 m	OM331729	ON934019	OP186287
34	Hm-110b-1	<i>D. rudis</i>	2021	Rodello (CN)	600 m	OM331735	ON934020	OP186288
35	HM-112a-1	<i>D. eres</i>	2021	Rodello (CN)	600 m	OM331730	ON934021	OP186289
36	HM-115b-3	<i>D. eres</i>	2021	Rodello (CN)	600 m	OM331731	ON934022	OP186290
37	Hm-128a-2	<i>D. novem</i>	2021	Cavaglia (BI)	270 m	OM331737	ON933988	OP156877
38	Hm-129a-2	<i>D. novem</i>	2021	Cavaglia (BI)	270 m	OM331736	ON933989	OP156878
39	HBr-108b-2	<i>D. foeniculina</i>	2021	Cavaglia (BI)	270 m	ON911321	ON934023	OP186291
40	HM-137a-1	<i>D. eres</i>	2021	Borgo D'Ale (VC)	240 m	OM331732	-	OP186292

<sup>1</sup> AL: Alessandria, CN: Cuneo, BI: Biella, VC: Vercelli; <sup>2</sup> ITS: internal transcribed spacers 1 and 4 together with 5.8S nrDNA; *tef1- $\alpha$* : translation elongation factor 1- $\alpha$  gene; *tub2*: beta-tubulin gene. Sequences generated in this study indicated in italics.

**Table S2.**

List of reference sequences of *Diaporthe* spp. including the outgroup *Diaporthella corylina* used in phylogenetic analyses for this study. Information includes GenBank accession numbers, strains, host, and origins.

Species	Strain <sup>1</sup>	Host	Country	GenBank Accession <sup>2</sup>		
				ITS	<i>tef1-a</i>	<i>tub2</i>
<i>Diaporthe ambigua</i>	CBS 114015 <sup>T</sup>	<i>Pyrus communis</i>	South Africa	KC343010	KC343736	KC343978
<i>D. ambigua</i>	CBS 123210	<i>Foeniculum vulgare</i>	Portugal	KC343012	KC343738	KC343980
<i>D. amygdali</i>	CBS 126679 <sup>T</sup>	<i>Prunus dulcis</i>	Portugal	KC343022	KC343748	KC343990
<i>D. amygdali</i>	CBS 115620	<i>Prunus persica</i>	USA	KC343020	KC343746	KC343988
<i>D. anacardii</i>	CBS 720.97 <sup>T</sup>	<i>Anacardium occidentale</i>	Eastern Africa	KC343024	KC343750	KC343992
<i>D. angelicae</i>	CBS 111592 <sup>T</sup>	<i>Heracleum sphondylium</i>	Austria	KC343027	KC343753	KC343995
<i>D. australafricana</i>	CBS 111886	<i>Vitis vinifera</i>	South Africa	KC343038	KC343764	KC344006
<i>D. australafricana</i>	CBS 113487 <sup>T</sup>	<i>Vitis vinifera</i>	South Africa	KC343039	KC343765	KC344007
<i>D. baccae</i>	CBS 136972 <sup>T</sup>	<i>Vaccinium corymbosum</i>	Italy	KJ160565	KJ160597	MF418509
<i>D. baccae</i>	CBS 142546	<i>Citrus sinensis</i>	Italy	MF418358	MF418437	MF418517
<i>D. beckhausii</i>	CBS 138.27	<i>Viburnum species</i>	Unknown	KC343041	KC343767	KC344009
<i>D. citri</i>	CBS 134239	<i>Citrus cinensis</i>	USA	KC357553	KC357522	KC357456
<i>D. citri</i>	CBS 135422 <sup>T</sup>	<i>Citrus</i> sp.	USA	KC843311	KC843071	KC843187
<i>D. cinerascens</i>	CBS 719.96	<i>Ficus carica</i>	Bulgaria	KC343050	KC343776	KC344018
<i>D. cucurbitae</i>	DAOM 42078	<i>Cucumis sativus</i>	Canada	KM453210	KM453211	KP118848
<i>D. cuppatea</i>	CBS 117499	<i>Aspalathus linearis</i>	South Africa	KC343057	KC343783	KC344025
<i>D. cynaroidis</i>	CBS 122676	<i>Protea cynaroides</i>	South Africa	KC343058	KC343784	KC344026
<i>D. eres</i>	CBS 138594 <sup>T</sup>	<i>Ulmus laevis</i>	Germany	KJ210529	KJ210550	KJ420799
<i>D. eres</i>	CBS 143344	<i>Vitis vinifera</i>	Czech Republic	MG281020	MG281541	MG281193
<i>D. eres</i>	CAA756	<i>Banksia</i> sp.	Portugal	MW040531	MW052385	MW091320
<i>D. eres</i>	CBS 146.46	<i>Alnus</i> sp.	The Netherlands	KC343008	KC343734	KC343976
<i>D. eres</i>	CBS 121004	<i>Juglans</i> sp.	USA	KC343134	KC343860	KC344102

<i>D. eres</i>	CBS 124030	<i>Malus pumila</i>	New Zealand	KC343149	KC343875	KC344117
<i>D. eres</i>	CBS 134470	<i>Castanea sativa</i>	Australia	KC343146	KC343872	KC344114
<i>D. eres</i>	DNP128	<i>Vaccinium corymbosum</i>	China	KC763096	KJ210561	KJ420801
<i>D. eres</i>	CBS 139.27	<i>Celastrus</i> sp.	USA	KC343047	KC343773	KC344015
<i>D. eres</i>	CBS 135428	<i>Juglans cinerea</i>	USA	KC843328	KC843121	KC843229
<i>D. eres</i>	CBS 439.82	<i>Cotoneaster</i> sp.	UK	KC343090	KC343816	KC344058
<i>D. eres</i>	CBS 338.89	<i>Hedera helix</i>	Yugoslavia	KC343152	KC343878	KC344120
<i>D. eres</i>	CBS 160.32	<i>Vaccinium macrocarpon</i>	USA	AF317578	GQ250326	KC344196
<i>D. eres</i>	MUM 19.31=CAA830	<i>Vaccinium corymbosum</i>	Portugal	MK792309	MK828080	MK837931
<i>D. foeniculina</i>	CBS 123208	<i>Foeniculum vulgare</i>	Portugal	KC343104	KC343830	KC344072
<i>D. foeniculina</i>	CBS 111553 <sup>T</sup>	<i>Foeniculum vulgare</i>	Spain	KC343101	KC343827	KC344069
<i>D. helianthi</i>	CBS 592.81 <sup>T</sup>	<i>Helianthus annuus</i>	Serbia	KC343115	KC343841	KC344083
<i>D. inconspicua</i>	CBS 133813 <sup>T</sup>	<i>Maytenus ilicifolia</i>	Brazil	KC343123	KC343849	KC344091
<i>D. infecunda</i>	CBS 133812 <sup>T</sup>	<i>Schinus terebinthifolius</i>	Brazil	KC343126	KC343852	KC344094
<i>D. lusitanicae</i>	CBS 123212	<i>Foeniculum vulgare</i>	Portugal	KC343136	KC343862	KC344104
<i>D. macadamiae</i>	BRIP 66526 <sup>T</sup>	<i>Macadamia</i> sp.	South Africa	MN708230	MN696528	MN696539
<i>D. malorum</i>	CAA740	<i>Malus domestica</i>	Portugal	KY435642	KY435629	KY435670
<i>D. malorum</i>	CBS 142383 <sup>T</sup>	<i>Malus domestica</i>	Portugal	KY435638	KY435627	KY435668
<i>D. nothofagi</i>	BRIP 54801	<i>Nothofagus cunninghamii</i>	Australia	JX862530	JX862536	KF170922
<i>D. novem</i>	CBS 127270	<i>Glycine max</i>	Croatia	KC343156	KC343882	KC344124
<i>D. novem</i>	CBS 127271 <sup>T</sup>	<i>Glycine max</i>	Croatia	KC343157	KC343883	KC344125
<i>D. novem</i>	CPC 26188 =CBS 142553	<i>Citrus japonica</i>	Italy	MF418426	MF418505	MF418586
<i>D. novem</i>	CPC 28165 = CBS 142554	<i>glycine max</i>	Italy	MF418427	MF418506	MF418587
<i>D. novem</i>	CPC 28167	<i>Citrus aurantiifolia</i>	Italy	MF418428	MF418507	MF418588
<i>D. oncostoma</i>	CBS 589.78	<i>Robinia pseudoacacia</i>	France	KC343162	KC343888	KC344130
<i>D. oncostoma</i>	CBS 100454	<i>Robinia pseudoacacia</i>	Germany	KC343160	KC343886	KC344128
<i>D. parvae</i>	PSCG 034 <sup>T</sup>	<i>Pyrus bretschneideri</i>	China	MK626919	MK654858	MK691248
<i>D. phaseolorum</i>	CBS 139281=AR4203	<i>Phaseolus vulgaris</i>	USA	KJ590738	KJ590739	KJ610893

<i>D. phillipsii</i>	MUM 19.28 <sup>T</sup>	<i>Vaccinium corymbosum</i>	Portugal	MK792305	MK828076	MN000351
<i>D. portugallica</i>	CBS 144228=CPC34247 <sup>T</sup> CBS 101339=	<i>Camelia sinensis</i>	Portugal Dominican	MH063905	MH063911	MH063917
<i>D. pseudomangiferae</i>	MFLU 15-3228 <sup>T</sup>	<i>Mangifera indica</i>	Republic	KC343181	KC343907	KC344149
<i>D. pterocarp</i>	MFLUCC 10-0571	<i>Pterocarous indicus</i>	Thailand	JQ619899	JX275416	JX275460
<i>D. ravennica</i>	MFLUCC 17-1029	<i>Salvia</i> sp.	Italy	KY964191	KY964147	KY964075
<i>D. ravennica</i>	MFLUCC 15-0479 <sup>T</sup>	<i>Tamarix</i> sp.	Italy	KU900335	KX365197	KX432254
<i>D. ravennica</i>	MFLUCC 15-0480	<i>Tamarix</i> sp.	Italy	KU900336	KX426703	KX377688
<i>D. rudis</i>	CBS 114436	<i>Sambucus cf. racemosa</i>	Sweden	KC343236	KC343962	KC344204
<i>D. rudis</i>	CBS 109492	<i>Laburnum anagyroides</i>	Austria	KC343232	KC343958	KC344200
<i>D. rudis</i>	CBS 113201 <sup>T</sup>	<i>Vitis vinifera</i>	Portugal	KC343234	KC343960	KC344202
<i>D. rudis</i>	CAA949	<i>Pinus pinaster</i>	Portugal	MN190304	MT309426	MT309452
<i>D. rudis</i>	CAA952	<i>Eucalyptus globulus</i>	Portugal	MN190307	MT309429	MT309455
<i>D. salicicola</i>	BRIP 54825	<i>Salix purpurea</i>	Australia	JX862531	JX862537	KF170923
<i>D. schini</i>	CBS 133181 <sup>T</sup>	<i>Schinus terebinthifolius</i>	Brazil	KC343191	KC343917	KC344159
<i>D. sennicola</i>	CFCC 51634	<i>Senna bicapsularis</i>	China	KY203722	KY228883	KY228889
<i>D. sojae</i>	CBS 139282=FAU635 <sup>T</sup>	<i>Glycine max</i>	USA	KJ590719	KJ590762	KJ610875
<i>D. subcylindrospora</i>	MFLU 17-1195	<i>Salix</i> sp.	China	MG746629	MG746630	MG746631
<i>D. terebinthifolii</i>	CBS 133180 <sup>T</sup> CGMCC	<i>Schinus terebinthifolius</i>	Brazil	KC343216	KC343942	KC344184
<i>D. velutina</i>	3.18286=LC4421 <sup>T</sup>	<i>Neolitsea</i> sp.	China	KX986790	KX999182	KX999223
<i>D. zaobaisu</i>	PSCG031 <sup>T</sup>	<i>Pyrus bretschneideri</i>	China	MK626922	MK654855	MK691245
<i>Diaporthella corylina</i>	CBS 121124 <sup>T</sup>	<i>Corylus</i> sp.	China	KC343004	KC343730	KC343972

<sup>1</sup> BRIP: Plant Pathology Herbarium, Department of Primary Industries, Dutton Park, Queensland, Australia CAA: Culture Collection Artur Alves, University of Aveiro, Aveiro, Portugal; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Beijing, China; CGMCC: China, General Microbiological Culture Collection, Beijing, China; CMW: Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute; DAOMC: The Canadian Collection of Fungal Cultures, Canada; DNP: Isolates in SMML culture collection, USDA-ARS, Beltsville, USA; FAU: Isolates in culture collection of Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville, MD, USA; LC: Working

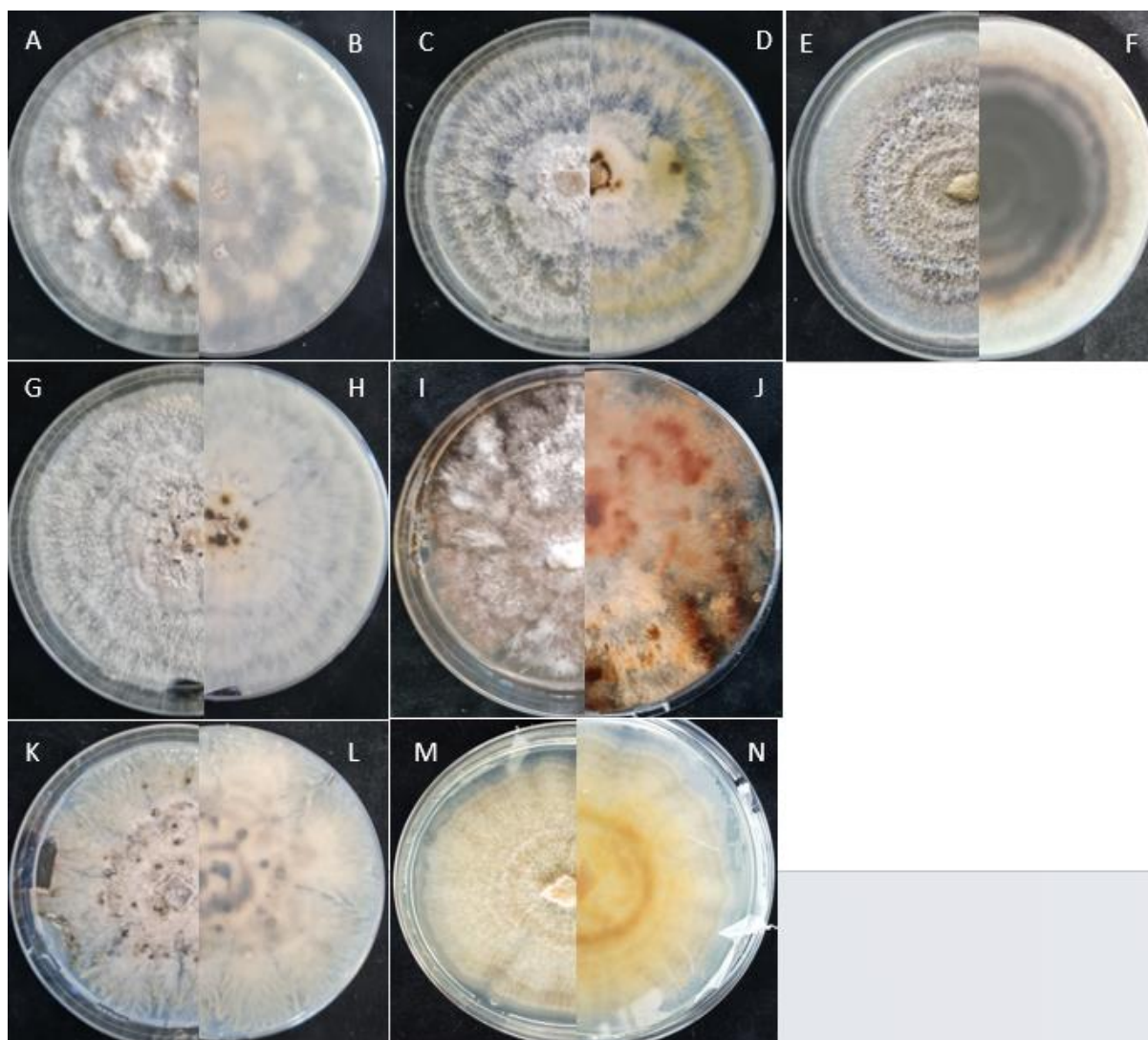
collection of Lei Cai, housed at Institute of Microbiology, Chinese; MFLU: Herbarium of Mae Fah Luang University, Thailand; MIFCC: Michigan Isolate Fungal Culture Collection, Michigan, USA; MUM: Culture Collection from Micoteca da Universidade do Minho, Center for Biological Engineering of University of Minho, Braga, Portugal. Ex-type isolates are indicated with <sup>T</sup>.

<sup>2</sup> ITS: internal transcribed spacers 1 and 4 together with 5.8S nrDNA; *tefl-α*: translation elongation factor 1-α gene; *tub2*: beta-tubulin gene. Sequences generated in this study indicated in italics.

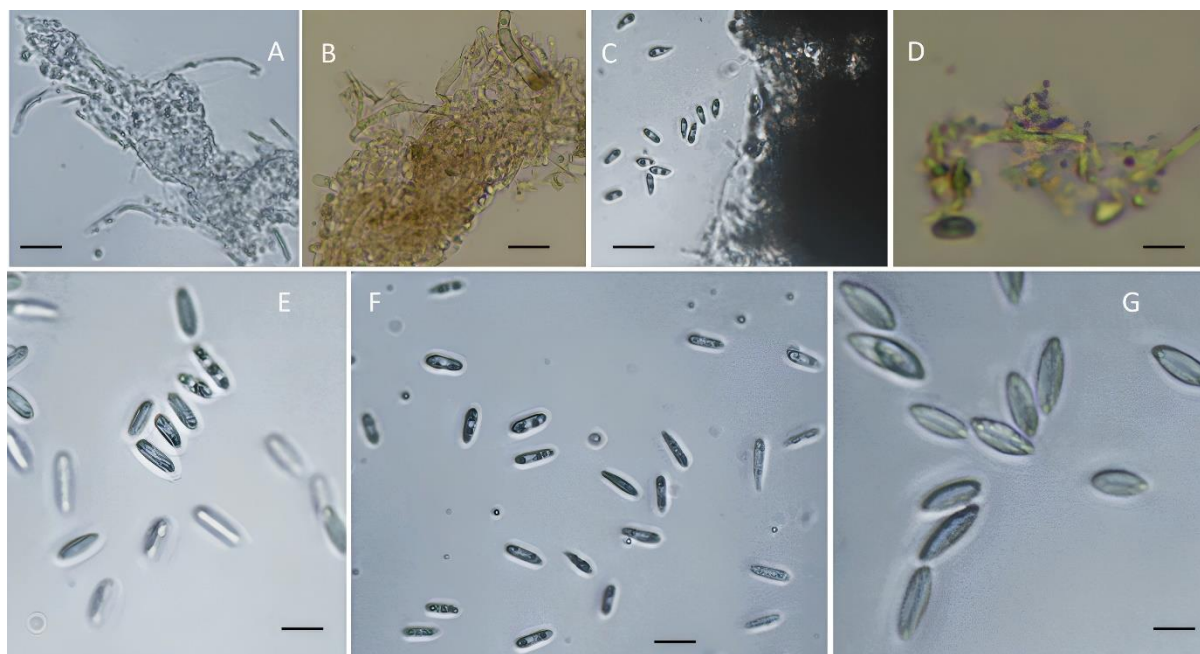
**Table S3.** Pathogenicity test of *Diaporthe* spp. on hazelnut nuts (*Corylus avellana*) and their disease severity

Sr. no.	Isolates code	Fungal spp.	DS% <sup>1</sup>	SD <sup>2</sup>	TK <sup>3</sup>
1	Hwa-18-1	<i>Diaporthe eres</i>	86.1	4.81	efg
2	HsN5a-2	<i>D. eres</i>	80.6	9.62	defg
3	HsM2a-3	<i>D. eres</i>	77.8	4.81	defg
4	HMb-1b-1	<i>D. eres</i>	83.3	8.33	efg
5	HMb-5a-3	<i>D. eres</i>	100.0	0.00	g
6	Hma-10a-1	<i>D. eres</i>	80.6	4.81	defg
7	HM-2a-1	<i>D. eres</i>	75.0	8.33	cdefg
8	HM-4b-2	<i>D. eres</i>	91.7	8.33	fg
9	HM-5a-1	<i>D. eres</i>	80.6	9.62	defg
10	HM-8b-1	<i>D. eres</i>	83.3	8.33	efg
11	HM-11b-2	<i>D. eres</i>	88.9	9.62	efg
12	Hm-12a-1	<i>D. novem</i>	77.8	9.62	defg
13	HM-17a-1	<i>D. eres</i>	97.2	4.81	fg
14	Hm-20a-2	<i>D. oncostoma</i>	36.1	4.81	abcd
15	HBR-2b-1	<i>D. eres</i>	52.8	4.81	bcdef
16	HBr-3a-2	<i>D. eres</i>	55.6	9.62	bcdefg
17	HBR-8a-1	<i>D. eres</i>	86.1	9.62	efg
18	HBr-8b-2	<i>D. rudis</i>	86.1	4.81	efg
19	HBr-9b-1	<i>D. ravennica</i>	13.9	9.62	ab
20	HBR-12b-1	<i>D. eres</i>	88.9	12.73	efg
21	HBr-13a-2	<i>D. foeniculina</i>	72.2	4.81	cdefg
22	HBR-15b-1	<i>D. eres</i>	88.9	9.62	efg
23	HBR-16a-1	<i>D. eres</i>	58.3	8.33	bcdefg
24	HN-27a-2	<i>D. eres</i>	86.1	9.62	efg
25	HBr-27a-2	<i>D. rudis</i>	13.9	4.81	ab
26	Hm-30-2	<i>D. sojae</i>	91.7	8.33	fg
27	HN-31a-2	<i>D. eres</i>	69.4	12.73	cdefg
28	HM-43b-1	<i>D. eres</i>	88.9	12.73	efg
29	Hm-46a-1	<i>D. foeniculina</i>	77.8	12.73	defg
30	HM-93a-1	<i>D. eres</i>	94.4	4.81	fg
31	HN-100a-2	<i>D. eres</i>	58.3	8.33	bcdefg
32	HM-108b-2	<i>D. eres</i>	58.3	8.33	bcdefg
33	HN-104a-2	<i>D. eres</i>	80.6	4.81	defg
34	Hm-110b-1	<i>D. rudis</i>	94.4	4.81	fg
35	HM-112a-1	<i>D. eres</i>	44.4	9.62	abcde
36	HM-115b-3	<i>D. eres</i>	86.1	12.73	efg
37	Hm-128a-2	<i>D. novem</i>	30.6	4.81	abc
38	Hm-129a-2	<i>D. novem</i>	75.0	0.00	cdefg
39	HBr-108b-2	<i>D. foeniculina</i>	100.0	0.00	g
40	HM-137a-1	<i>D. eres</i>	75.0	0.00	cdefg
41	Healthy Control		0.00	0.00	a

<sup>1</sup> Disease severity in percentage <sup>2</sup> standard deviation <sup>3</sup> Tukey test



**Fig. S1.** Macromorphology of *Diaporthe* spp. obtained from hazelnut nuts **(A-B)** *D. sojae*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C, **(C-D)** *D. foeniculina*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C, **(E-F)** *D. novem*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C, **(G-H)** *D. ravennica*. Front and reverse side of colony grown on PDA at  $23 \pm 2$  °C, **(I-J)** *D. rudis*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C, **(K-L)** *D. eres*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C, **(M-N)** *D. oncostoma*. Front and reverse side of colony grown on PDA after 10 days at  $23 \pm 2$  °C.



**Fig. S2.** Micromorphological features of *Diaporthe* spp. obtained from hazelnut nuts **(A)** Conidia of *D. sojae* (dimensions 5.8-7.3x2-3.2; scale bar: 10  $\mu$ m), **(B)** Conidia of *D. foeniculina* (dimensions 7.6-9.5x2.5-2.9  $\mu$ m; scale bar: 10  $\mu$ m), **(C)** Conidia of *D. novem* (dimensions: 6.9-9.1x2.5-2.9  $\mu$ m; scale bar: 10  $\mu$ m), **(D)** Conidia of *D. ravennica* (dimensions 8.3-11.9x2.5-4.3  $\mu$ m; scale bar: 10  $\mu$ m), **(E)** Conidia of *D. rudis* (dimensions: 7.5-8.5x2.4-2.7  $\mu$ m; scale bar: 10  $\mu$ m), **(F)** Conidia of *D. eres* (dimensions: 8.5-11.1x 3.5-4.5  $\mu$ m; scale bar: 10  $\mu$ m), **(G)** Conidia of *D. oncostoma* (11-14.5x 3.2-4.9  $\mu$ m; scale bar: 10  $\mu$ m).