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UNIVERSITÀ DEGLI STUDI DI TORINO

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Abstract

In the last decades, productivity of Italian chestnut groves has been seriously impacted by the infection of the emerging fungal pathogen *Gnomoniopsis castaneae*, causing nut rots. Despite the widespread distribution of this pathogen in Italy, its genetic diversity and phylogeographic structure is still largely unexplored. In this study, we analysed the genetic diversity and spatial distribution of *G. castaneae* haplotypes by investigating 108 isolates from the northwest, the northeast and central Italy, as well as Sardinia. In Sardinia, where little was known about the occurrence of *G. castaneae* as a nut rot agent, a thorough investigation was conducted in three sites on a total of 1500 nuts. Phylogenetic analyses of sequences of β-tubulin revealed the occurrence at a worldwide scale of two distinct evolutionary lineages (here reported as haplotype A and B), with different frequencies in Italy depending on the geographic area. Based on the outcomes of inoculation experiments on nuts, both haplotypes proved to be pathogenic, although with marked differences in aggressiveness.

Keywords: emerging pathogen; haplotypes; phylogeographic pattern; pathogenicity

Over the past century, sweet chestnut (*Castanea sativa* Mill.) has been greatly impacted by several exotic and invasive insect pests and fungal pathogens (Vettraino et al. 2005; Gonthier and Robin, 2020; Fernandes et al. 2022), suggesting a high vulnerability of this multipurpose tree species to biotic threats. Since the mid-2000s, an increase in chestnut nut rots, hereafter called chestnut brown rot (CBR), caused by the fungal pathogen *Gnomoniopsis castaneae* G. Tamietti was observed across America, Asia, Australasia and Europe, making this disease a major global threat to chestnut systems growers (Visentin et al. 2012; Lione et al. 2019; Dobry and Campbell, 2023). The first reports of the disease occurred in north-western Italy and Australia (Visentin et al. 2012; Shuttleworth et al. 2013). While the disease is well known to chestnut nut growers in most of Italy (Maresi et al., 2013; Vettraino et al. 2021), there is still a gap of knowledge on its presence in Italian islands, including Sardinia (Seddaiu et al. 2017). Furthermore, little is known on the genetic variability of populations of *G. castaneae* throughout Italy. The only information available on this subject, to date, refers to a study

conducted by using SSR markers and involving *G. castaneae* isolates from north-western Italy, as well as other isolates from southern European countries (Sillo et al. 2017). Based on that study, as outlined by several approaches, including analysis of shared haplotypes, multivariate and Bayesian analyses, at least two putative distinct subpopulations of the fungus were identified (Sillo et al. 2017). The evolutionary and geographic origins of *G. castaneae* and the pathways of its spread are unknown. Nevertheless, it could be hypothesised that in Europe the pathogen introduction in new countries overlapped with that of chestnut trees spread all over Europe throughout the Roman empire. While the population diversity of *G. castaneae* and its geographic distribution is still poorly understood, the knowledge of the phenotypic and genetic structure of pathogen populations is fundamental for understanding the mechanisms generating genetic variation and host-pathogen interaction, and for developing effective management control strategies. The main purposes of this study were: 1) to evaluate the occurrence of *G. castaneae* in chestnut orchards in Sardinia, and 2) to study the occurrence and frequency of haplotypes of *G. castaneae* throughout Italy, including areas from northwest, northeast, central Italy, as well as from Sardinia.

In 2019, 1500 chestnuts were randomly collected in three of the main chestnut growing areas in Sardinia (Italy) and promptly processed to ascertain the occurrence of *G. castaneae* infections (Table 1). At each site the chestnuts were obtained from fallen fruits and from nuts still in the burr and attached to the tree in equal parts.

Table 1 Details of surveyed sites in Sardinia and number of samples collected

Study Sites	Elevation (m a.s.l.)	Locality	Geographic Coordinates		Management	Number of Samples
A	990	Aritzo	39°56′55” N	09°11′45” E	Semi natural	600 (N)
B	936	Belvì	39°58’20” N	09°09′41” E	Semi natural	600 (N)
C	520	Tempio Pausania	40°52’50” N	09°08’45” E	Semi natural	300 (N)

Nuts were firstly washed under tap water, then disinfected in 70% ethanol for 1 min and air-dried in aseptic conditions. Chestnuts were cut in half and visually assessed for the presence of alteration of

the endosperm such as necrotic lesions or brown rot symptoms. Chestnut tissues were cut into small segments (approximately 5 mm^2), and the fungal community was isolated on PDA as described by Vettraino et al. (2021). The fungal isolates were initially grouped in morphotypes based on colony appearance and conidia morphology.

Molecular analysis was used to confirm identification of all isolates to species level. DNA was extracted from 5-day-old cultures grown on PDA and incubated at 25 °C in the dark using the InstaGene Matrix (BioRad Laboratories, Hercules, CA). For all isolates the ITS region was amplified and sequenced with the primers ITS1 and ITS4 (White et al. 1990), while the primers Bt2a/Bt2b (Glass and Donaldson 1995) were used to amplify and sequence a portions of the β-tubulin (tub2) region of a representative set of *G. castaneae* isolates. PCR mixtures and amplification conditions were as described by Linaldeddu et al. (2016). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA) and then compared with reference sequences (ex-type culture or representative strains) retrieved in GenBank using the BLAST search function. Isolates were assigned to a species when their sequences were at least 99.8% identical to the sequence of type material or representative isolates. The ITS and β-tubulin sequences of seven representative isolates of *G. castaneae* obtained in this study were deposited in GenBank (accession numbers: MN809478 to MN809496 and MN817091 to MN817109 for ITS and β-tubulin regions respectively). The phylogenetic relationships and haplotype diversity of *G. castaneae* isolates were examined by analyzing the β-tubulin sequences (450 bp) of 108 isolates from six Italian regions: Sardinia (19 isolates), Latium (45) (central Italy), Piedmont and Aosta Valley (5) (north-western Italy), Veneto (32) and Trentino Alto Adige (6) (north-eastern Italy). Among them, β-tubulin sequences of 4 isolates chosen as representative of the Italian population, were compiled in a dataset including sequences of other 21 *G. castaneae* isolates downloaded from GenBank and the outgroup *Gnomoniopsis sanguisorbae* (Table 2).

Table 2 Details of *Gnomoniopsis castaneae* isolates included in the phylogenetic analyses.

Isolate name	Strain	Host	Location	ITS	B-tubulin
G. castaneae	TAV_1_1	*C. sativa*	Switzerland	KM437892	KM437888
G. castaneae	BIA_1_1	*C. sativa*	Switzerland	-	KM437889
G. castaneae	CAS_1_1	*C. sativa*	Switzerland	-	KM437890
G. smithogilvyi	Ti1	*C. sativa*	Switzerland	KP824746	KP824764
G. smithogilvyi	Ti3	*C. sativa*	Switzerland	KP824748	KP824765
G. smithogilvyi	Ti4	*C. sativa*	Switzerland	KP824750	KP824766
G. smithogilvyi	Ti5	*C. sativa*	Switzerland	KP824752	KP824767
G. smithogilvyi	Ge1	*C. sativa*	Switzerland	KP824754	KP824768
G. smithogilvyi	MUT401	*C. sativa*	Italy	HM142946	KR072532
G. smithogilvyi	MUT411	*Castanea* sp.	New Zealand	HM1142948	KR072533
G. smithogilvyi	GCAS1	*C. sativa*	Greece	-	MH213477
G. *castaneae*	GCAS2	*C. sativa*	Greece	-	MH213478
G. castaneae	GCAS3	*C. sativa*	Greece	-	MH213479
G. castaneae	GCAS4	*C. sativa*	Greece	-	MH213480
G. castaneae	GCAS5	*C. sativa*	Greece	-	MH213481
G. castaneae	E2	*C. sativa*	Italy	MN809483	MN817096*
G. castaneae	E3	*C. sativa*	Italy	-	MN817097*
G. castaneae	E4	*C. sativa*	Italy	MN809485	MN817098*
G. castaneae	E5	*C. sativa*	Italy	MN809486	MN817099*
G. castaneae	UP45119	*Castanea* sp.	USA	MZ681935	OK335785
G. castaneae	PSB3	*Castanea* sp.	USA	MZ682108	OK335787
G. castaneae	EFA924A	*C. sativa*	Spain	OM319846	OM417078
G. smithogilvyi	EFA925A	*C. sativa*	Spain	OM319847	OM417079
G. smithogilvyi	EFA962.4A	*C. sativa*	Spain	OM319848	OM417080
G. smithogilvyi	Gc_01	*C. sativa*	Turkey	ON326601	ON337137
G. sanguisorbae	CBS 858.79	*C. sativa*	USA	GU320818	GU320790

*indicates the isolates chosen as representative of the Italian population and reported in the Neighbour-joining tree

Sequences were aligned with Clustal X 1.83 (Thompson et al. 1997). The evolutionary history was inferred using the Neighbor-Joining method (Saito et al., 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of

base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA11 (Tamura et al 2021).

Seven representative Sardinian isolates, 4 belonging to the haplotype A and 3 to the haplotype B, were selected to evaluate their virulence on nuts. A total of 200 chestnuts, collected in the Gennargentu area (Desulo, Sardinia) were washed, sterilised, weighted and visually assessed for the presence of brown rot symptoms. Finally, a total of 84 asymptomatic chestnuts were randomly selected to be used in the tests. Treated nuts were inoculated with an agar-mycelium plug (8 mm^2) cut from the margin of the *G. castaneae* isolates (5-day-old PDA). The inoculation site was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminium foil. Sterile PDA plugs were used for control treatments (10 nuts). Chestnuts were kept at 22 °C in the dark for 18 days. At the end of the experimental period, chestnut symptomatic tissues were removed and weighted. *Gnomoniopsis castaneae* was re-isolated from the margin of chestnut lesions.

Statistical analyses were performed using XLSTAT software (Addinsoft, France). The Student's t-test was used to detect differences in virulence between haplotypes at $P = 0.05$. Geographic distribution of the two haplotypes of *G. castaneae* according to their origins in northern and central Italy were analyzed by Chi-squared test of a 2×2 contingency table.

Both fallen ripened nuts (32%) and nuts in the burr (29%) showed typical CBR symptoms consisting in a progressive alteration of colour and consistency of endosperm tissues conferring the appearance of a "grey mummy", and black rot of the internal tissues. The CBR incidence was not affected by the sites (Chi-square: $\chi^2 = 1.99$, $p>0.05$) and ranged from 10% up to 32%. The fungal microbiome isolated from chestnut tissues was assigned to different genera and species, based on the morphological features and DNA sequences data. A total of 11 species were identified: *Alternaria alternata* (2 isolates), *Botrytis cinerea* (3), *Cladosporium* sp. (1), *Cryphonectria* sp. (1), *G. castaneae* (125), *Mucor fragilis* (8), *Neofusiccocum parvum* (15), *Penicillium* sp. (51), *Rutstroemia echinophila* (55), *Talaromyces* sp. (3), *Trichoderma* sp. (1). *Gnomoniopsis castaneae* was the most frequently isolated

fungal species. Occurrence of this pathogen was higher on symptomatic chestnuts (95 isolates, 76 %) than apparently healthy chestnuts (30 isolates, 24%) (χ^2 = 33.8, p<0.0001).

Alignment of ITS sequences data of all *G. castaneae* isolates did not allow the detection of any DNA polymorphisms, while alignment of β-tubulin sequences revealed the occurrence of four fixed polymorphisms among isolates and therefore, the existence of two haplotypes here reported as A and B (Fig. 1). The phylogenetic analysis based on sequences of the tub2 gene region showed that the *G. castaneae* isolates from different countries clustered within two subclades representing the two haplotypes (Fig. 1).

The 108 Italian isolates of *G. castaneae* clustered into both haplotypes. In particular, 52% of the Italian isolates belonged to the haplotype A and 48% to the haplotype B. Analysis of the geographic distribution of Italian isolates revealed that haplotypes and regions were not independent. Therefore, the two haplotypes are not distributed equally in the two major geographic areas studied. Haplotype A was dominant in central Italy and haplotype B in northern Italy (Fig. 2) and (Table 3).

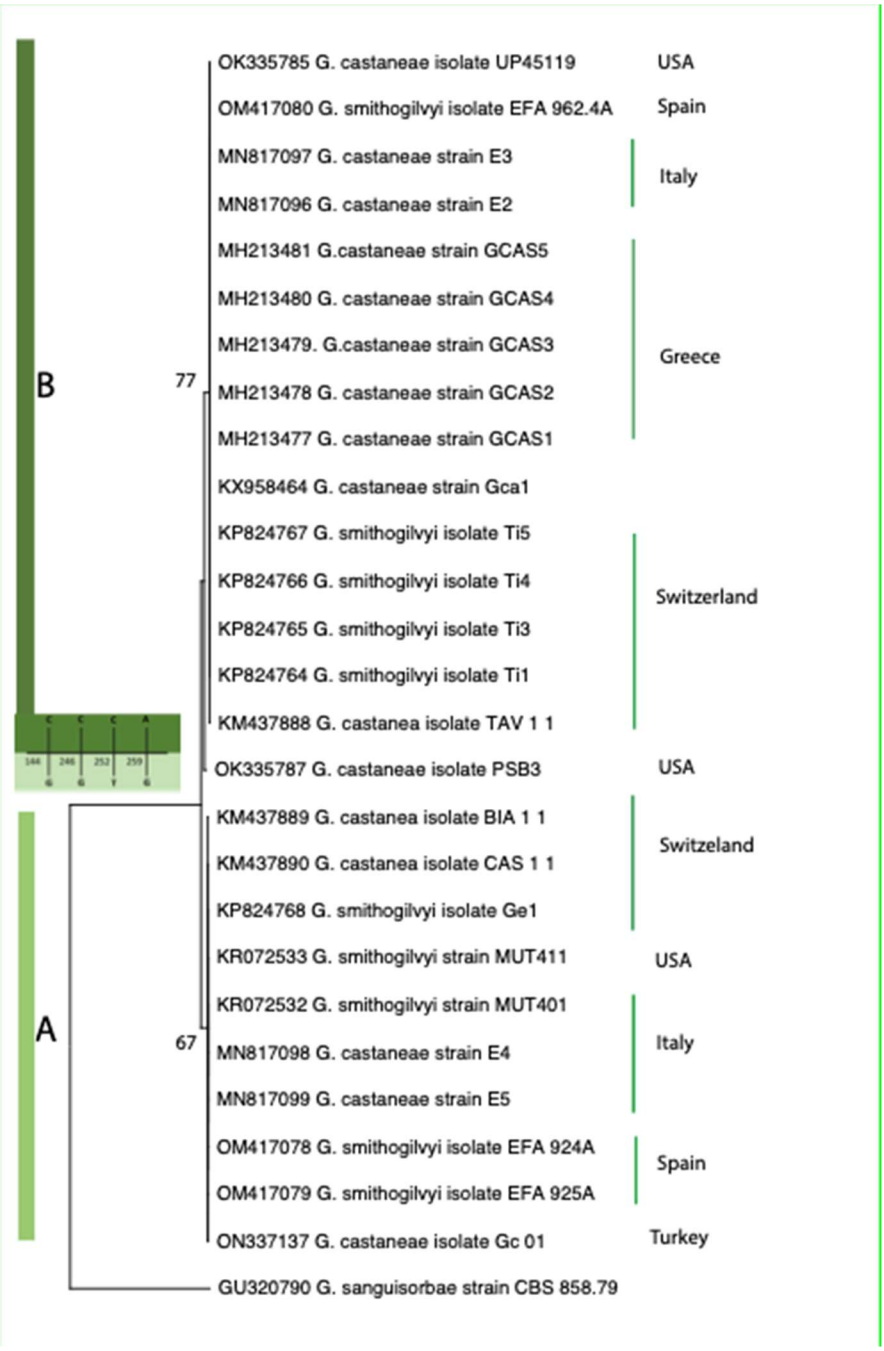

Fig. 1 Neighbour-joining tree based on *Gnomoniopsis castaneae* β-tubulin sequences from isolates of different countries. Bootstrap support values in percentage are given at the nodes. The scale bar represents the number of substitutions per site. Two different haplotypes (A and B) within *G. castaneae* are indicated. In the green box are reported the polymorphic nucleotides from aligned sequence data of β-tubulin region showing the variation among *G. castaneae* isolates.

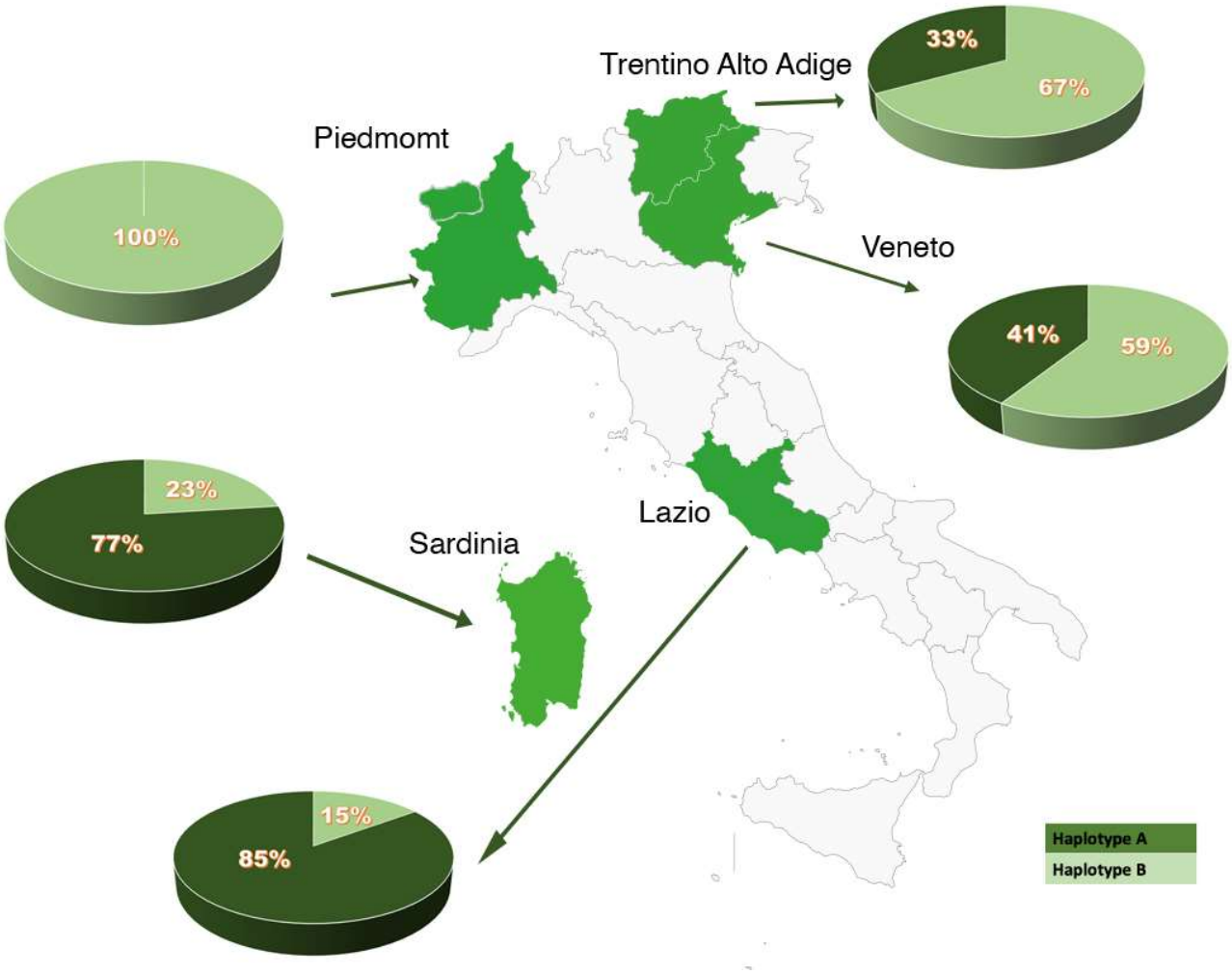

Fig. 2 Geographic distribution of haplotypes A and B of *Gnomoniopsis castaneae* in Italy.

Table 3 Distribution of haplotype A and B of *Gnomoniopsis castaneae* according to their origins in Northern Italy (Piedmont, Veneto, Trentino-Alto Adige) or central Italy (Sardinia and Lazio)

	Lineage A	Lineage B	Chi-square
Northern Italy			$\chi^2 = 55{,}125$, ($p<0.0001$)
Central Italy			$\chi^2 = 21{,}294$, ($p<0.0001$)

Gnomoniopsis castaneae isolates of both haplotypes A and B were pathogenic to chestnut causing CBR symptoms congruent with field observations. *Gnomoniopsis castaneae* was re-isolated from all inoculated samples, thus fulfilling Koch's. Controls were visually asymptomatic and *G. castaneae* negative. The haplotype A showed to be the most virulent, with a mean value of the symptomatic area of 3.04 ± 0.37 mm^2, value significantly higher than that of the haplotype B 1.93 ± 0.24 mm^2 (Student's T-test: valore, $p<0.05$).

This study represents the most comprehensive investigations on the phylogeographic diversity of *G. castaneae* isolates in Italy. In accordance with Dennert et al. (2015) our results showed that β-tubulin sequences *G. castaneae* are not identical, allowing to separate the isolates in two distinct lineages that can be referred to haplotypes A and B. Whether these two haplotypes may reflect the two putative subpopulations described by Sillo et al. (2017) on the basis of a SSR genotyping, remains an open question because all isolates from north-western Italy included in this study, although coming from different sites, belonged to the haplotype B. The discovery of two haplotypes with a different geographic distribution suggests the need to extend the phylogeographic studies on *G. castaneae* in order to identify the most prevalent evolutionary lineage at local and regional scale in the chestnut growing areas in Europe. The occurrence of genetic diversity can also indicate greater ability to adapt to changing environmental conditions and may reflect increased biological fitness of these genotypes. Existence of intraspecific variability in housekeeping genes encoding β-tubulin or elongation factor alpha (EF-1α) has recently been reported for several fungal pathogens such as *Seiridium cardinale* (della Rocca et al., 2011), *Sardiniella urbana* (Linaldeddu et al., 2016) and *Diplodia corticola* (Smahi et al., 2017). The intraspecific variability of these housekeeping genes raises many questions about how the molecular data should be analysed and the potential limits posed by multi-loci sequence analysis (MLSA) in the correct species delimitation. The choice of appropriate loci that optimizes species separation is often difficult, for *G. castaneae* the β-tubulin locus should be excluded in MLSA.

Finally, the discovery of several fungal species associated with CBR symptoms poses an additional threat to chestnut stands in Italy. In particular, the occurrence of *N. parvum* on chestnut is of great concern due to its wide host range and the impact of this pathogen on forest ecosystems (Manca et al., 2020).

References

Della Rocca G, Eyre CA, Danti R, Garbelotto M (2011) Sequence and simple-sequence repeat analyses of the fungal pathogen *Seiridium cardinale* indicate California is the most likely source of the cypress canker epidemic for the Mediterranean region. Phytopathology 101:1408–1417.

Dennert F, Broggini G, Gessler C, Storari M (2015) *Gnomoniopsis castanea* is the main agent of chestnut nut rot in Switzerland. Phytopathol. Mediterr. 54(2):199–211

Fernandes P, Colavolpe MB, Serrazina S, Costa RL (2022) European and American chestnuts: DOIAn overview of the main threats and control efforts. Front Plant Sci 13:951844

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330

Gonthier P, Robin C (2020) Diseases. Chapter 11. In: The Chestnut Handbook: Crop and Forest Management, Beccaro G, Alma A, Bounous G, Gomes-Laranjo J (Eds). CRC Press, Taylor & Francis Group, Boca Raton, FL, USA. pp. 297–316

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111–120

Linaldeddu BT, Alves A, Phillips AJL (2016) *Sardiniella urbana* gen. et sp. nov., a new member of the *Botryosphaeriaceae* isolated from declining *Celtis australis* trees in Sardinian streetscapes. Mycosphere 7:893–905

Lione G, Danti R, Fernandez-Conradi P, Ferreira-Cardoso JV, Lefort F, Marques G, Meyer JB, Prospero S, Radócz L, Robin C, Turchetti T, Vettraino AM, Gonthier P (2019) The emerging

pathogen of chestnut *Gnomoniopsis castaneae*: the challenge posed by a versatile fungus. Eur J Plant Pathol 153:671–685

Manca D, Bregant C, Maddau L, Pinna C, Montecchio L, Linaldeddu B (2020). First report of canker and dieback caused by *Neofusicoccum parvum* and *Diplodia olivarum* on oleaster in Italy. Ital J Mycol 49(1):85–91.

Maresi G, Oliveira Longa CM, Turchetti T (2013) Brown rot on nuts of *Castanea sativa* Mill: an emerging disease and its causal agent. iForest 6:294–301

Saito N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425

Seddaiu S, Cerboneschi A, Sechi C, Mello A (2017) *Gnomoniopsis castaneae* associated with *Dryocosmus kuriphilus* galls in chestnut stands in Sardinia (Italy). iForest 10:440–445

Shuttleworth LA, Liew ECY, Guest DI (2013) Survey of the incidence of chestnut rot in south-eastern Australia. Australasian Plant Pathology 42: 63–72

Sillo F, Giordano L, Zampieri E, Lione G, De Cesare S, Gonthier P (2017) HRM analysis provides insights on the reproduction mode and the population structure of *Gnomoniopsis castaneae* in Europe. Plant Pathology 66:293–303

Smahi H, Belhoucine-Guezouli L, Berraf-Tebbal A, Chouih S, Arkam M, Franceschini A, Linaldeddu BT, Phillips AJL (2017) Molecular characterization and pathogenicity of *Diplodia corticola* and other *Botryosphaeriaceae* species associated with canker and dieback of *Quercus suber* in Algeria. Mycosphere 8(2):1261–1272

Tamura K, Stecher G, and Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis version 11. Molecular Biology and Evolution 38:3022–3027

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876–4882

Vettraino AM, Morel O, Perlerou C, Robin C, Diamandis S, Vannini A (2005) Occurrence and distribution of *Phytophthora* species in European chestnut stands, and their association with Ink Disease and crown decline. Eur J Plant Pathol 111:169–180

Vettraino AM, Luchi N, Rizzo D, Pepori AL, Pecori F, Santini A (2021) Rapid diagnostics for *Gnomoniopsis smithogilvyi* (syn. *Gnomoniopsis castaneae*) in chestnut nuts: new challenges by using LAMP and real-time PCR methods. AMB Expr 11:105 https://doi.org/10.1186/s13568-021-01266-w

Visentin I, Gentile S, Valentino D, Gonthier P, Tamietti G, Cardinale F (2012) *Gnomoniopsis castanea* sp. nov. (Gnomoniaceae, Diaporthales) as a causal agent of nut rot in sweet chestnut. Journal of Plant Pathology 94:411–419

White T J, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal DNA for phylogenetics. In: "PCR Protocols: a guide to methods and applications" (Innis MA, Gelfand DH, Sninsky JJ, White TJ eds). Academic Press, San Diego, CA, USA, pp 315-322