

***GNOMONIOPSIS CASTANEA* sp. nov. (GNOMONIACEAE, DIAPORTHALES) AS THE CAUSAL AGENT OF NUT ROT IN SWEET CHESTNUT**

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SUMMARY

The genus *Gnomoniopsis* (Gnomoniaceae, Diaporthales) is currently composed of 13 species which are endophytic and/or parasitic to plants in the families Fagaceae, Onagraceae and Rosaceae. Species definition is based on a combination of morphological traits, association with specific plant hosts, and phylogeny. In this paper a new species, *Gnomoniopsis castanea* sp. nov., is described based on the association with *Castanea sativa* (a plant species never reported to be infected by fungi the genus *Gnomoniopsis*), morphology and phylogenetic analysis of the ITS region of ribosomal DNA and on the *EF1-a* locus. The fungus is consistently associated with nut rot and caused the disease when artificially inoculated to fruits or flowers. Infection incidence varied depending on the orchard and the year and attained up to 83% of the nuts in some areas of north-western Italy. The fungus was also consistently isolated from the bark of symptomless branches in naturally infected chestnut orchards.

Key words: DNA sequencing, endophyte, EF1- α , ITS, Koch's postulates, pathogen, taxonomy.

INTRODUCTION

The genus *Gnomoniopsis* includes endophytes and pathogens causing disease also in economically important plants such as blackberry, raspberry, strawberry (Bolay, 1971; Maas, 1998; Monod, 1983), *Quercus alba* and *Q. rubra* (Cohen, 2004; Sogonov *et al.*, 2007). In the past, *Gnomoniopsis* has often been considered a synonym of *Gnomonia* (Barr, 1978; Monod, 1983). In recent years, however, the analysis of host identity in combination with morphology and phylogeny has allowed the revival of this genus and the revision of its taxonomy. The comprehensive and recent definition of the biological, morphological and phylogenetic species con-

cepts for the genus *Gnomoniopsis* has led to a clearer delimitation of its borders within the genera of Gnomoniaceae. A recent revision of the genus has added to the three species (*G. chamaemori*, *G. clavulata* and *G. paracavulata*) originally proposed by Sogonov *et al.* (2008), 10 new members denoted *G. alderdunense*, *G. comari*, *G. fructicola*, *G. guttulata*, *G. idaeicola*, *G. macounii*, *G. occulta*, *G. racemula*, *G. sanguisorbae*, and *G. tormentillae* (Walker *et al.*, 2010), thus bringing to 13 the number of effectively, legitimately and validly published *Gnomoniopsis* species.

Fungi belonging to this genus have small, black perithecia, immersed in the host tissue as solitary structures lacking a stroma, or in small groups (up to 11) with minimal stroma. The neck is single, central, marginal or lateral; the asci are oval to fusiform and contain eight spores with an apical ring (Sogonov *et al.*, 2008; Walker *et al.*, 2010). Ascospores have a single sept and no appendages, with the exception of *G. tormentillae* which bears short appendages (Barr, 1978). The asexual stage produces subglobose pycnidia with hyaline, one-celled conidia of different shapes (oval, oblong, globose or femur-shaped). The association with host plants is genus-specific (mostly Fagaceae, Rosaceae and Onagraceae) and remains crucial for species definition. None of the *Gnomoniopsis* species described so far has been reported in association with sweet chestnut (*Castanea sativa* Mill.) (Walker *et al.*, 2010).

Chestnut is economically important as a landscape element, a tool for environment preservation and a source of timber and fruit. In Europe, it is present in the Mediterranean, Caucasian, and Balkan regions, Portugal and Southern England. In Italy, chestnut used to be of particular importance to the economy of some mountain communities until the middle of the 20th century; since then, as a result of a number of socio-economic and pathological factors, its importance has gradually declined. Nowadays there is a renewed demand for high quality chestnut nuts, complemented by active marketing efforts and by the development of mechanised harvesting and processing technologies (Bounous and Torello Marinoni, 2005). The quality of chestnut fruits can be affected in pre- and post-harvest by insects and moulds. Nut rot, one of the first described diseases was known in

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northern Italy since the second half of the 19th century as “chestnut mummification” (Spegazzini, 1879). Afterwards, the disease was investigated by Voglino and Bongini (1918) and Servazzi (1941), and attributed to *Phoma endogena* Spegazzini. In 1947, mummification of chestnuts was observed both in Viterbo (Latium, central Italy) and Cuneo (Piedmont, northern Italy). In those cases the causal agents were identified as *Phomopsis viterbensis* sp. nov., because of the presence of both α and β conidia (Camici, 1948). Ciferri (1950) identified a *Phomopsis* in sweet chestnut nut samples from Piedmont and, since he could observe stylospores (β conidia) in Voglino and Bongini’s specimen, he emended *Phomopsis viterbensis* to *Phomopsis endogena* (Speg.) nobis n. comb. More recent reports from Italy and other countries indicate the black rot fungus *Ciboria batschiana* (Zopf) Buchw., *Sclerotinia pseudotuberosa* Rehm., *Phomopsis castanea* (Sacc.) Petr. [tel. *Cryptodiaporthe castanea* (Tul. et C. Tul.) Wehm.], and *Phomopsis castaneae* (Moriondo) as causal agents of chestnut rot (Baudry and Robin, 1996; Breisch, 2008; Delatour and Morelet, 1979; Sieber *et al.*, 2007; Vettraino *et al.*, 2005; Wadia *et al.*, 1999; Washington *et al.*, 1997, 1999). In 2008, a new agent of chestnut nut rot in New Zealand was preliminarily identified as *Discula pascoe* sp. nov. (teleomorph: *Gnomonia pascoe* sp. nov.) (Smith and Agri, 2008) but was not validly published in a taxonomic sense (Seifert and Rossman, 2010). Since 2005, chestnut growers in north-western Italy have been suffering increasing yield losses, due to rotting of the ripe nuts both before and after picking. The disease was initially attributed to fungi commonly found on chestnut fruits, such as *P. castanea* or *C. batschiana*, but a more detailed analysis revealed the a previously undescribed fungal species as the main causal agent of rot. The present study reports its characterization as *Gnomoniopsis castanea* sp. nov.

MATERIALS AND METHODS

Specimen collection, fungal isolation methods, cultures and media. Samples consisting of a minimum of 24 mature chestnut fruits were collected in 2007, 2008, 2009 and 2011 in no less than five chestnut stands located in the municipalities of Boves, Peveragno, Robilante, Valdieri (Cuneo, north-western Italy). A minimum of 20 6- to 36-month-old branches were also sampled in each stand. On July 25, 2007 and July 30, 2010, samples of 100 burrs each fallen to the ground the previous years were collected in Robilante and Boves, respectively. Additional nut samples were collected in other areas of Piedmont, and were obtained from south-eastern France and southern Switzerland. In 2008, a disease very similar to that we observed in Cuneo was reported in New Zealand (Smith and Agri, 2008), so a fungal strain from New Zealand (NZ11) was obtained and included in our analyses (Table 1).

Isolations were made on malt extract agar (MEA) adjusted with citric acid to pH 4.65 (0.5 g l⁻¹) fruits or branches that were washed in tap water, disinfected in 2% sodium hypochlorite (5 min) and allowed to dry under a sterile air flow. Chestnut fruits were cut with a sterile scalpel, and small pieces of symptomatic or symptomless pulp were plated and incubated at 25°C for one week. Branches were cut into 1-2 mm thick transversal disks, the bark was separated from xylem and both tissues were plated. Emerging colonies were preliminarily identified based on the morphological characters of conidia and conidiophores or fruit bodies. A fast-growing fungus producing globose, pycnidium-like acervuli was largely predominating over the other fungi, and frequently it was the only one to develop. Representative colonies of this fungus were individually transferred onto MEA and used to produce single-spore isolates.

Table 1. Isolates sequenced in this work, with their MUT (Mycotheca Universitatis Taurinensis) and GenBank accession numbers.

ID	Locality	Tissue	Year	Accession numbers		
				MUT	ITS	EF1_
Rb7	Robilante (IT)	fruit	2007	MUT 401	HM1142946	JQ791198
VA8	Valdieri (IT)	fruit	2008	MUT 403	HM1142947	JQ791200
NZ11	New Zealand	fruit	2007	MUT 411	HM1142948	JQ791201
BvCo20	Boves (IT)	fruit	2007	MUT 418	HM1142950	JQ791202
BvCe29	Boves (IT)	branch	2008	MUT 452	HM1142955	JQ791203
Cv32	Ceva (IT)	fruit	2009	MUT 455	HM1142956	JQ791204
Pr36	Prarostino (IT)	fruit	2009	MUT 802	HM1142958	JQ791205
BS38	Bassa Val Susa (IT)	fruit	2009	MUT 806	HM1142959	JQ791206
VG40	Valgioie (IT)	fruit	2009	MUT 807	HM1142960	JQ791207
Sa42	Santena (IT)	fruit	2009	MUT 808	HM1142961	JQ791208
No44	Novara (IT)	fruit	2009	MUT 809	HM1142962	JQ791209
CH2	Biasca (CH)	fruit	2011	MUT 810	JQ898295	JQ791210
CH3	Biasca (CH)	fruit	2011	MUT 811	JQ898296	JQ791211
CH4	Cadenazzo (CH)	fruit	2011	MUT 814	JQ898297	JQ791199
FR	Sisteron (FR)	fruit	2011	MUT 815	JQ898298	JQ791212

Inoculation tests. Isolates Rb7 and VA24 (Table 1) were grown on MEA until actively sporulating; conidia were washed with sterile distilled water to obtain suspensions that were adjusted to 2×10^7 conidia ml⁻¹. Healthy chestnut nuts collected in the Susa Valley (Piedmont) were artificially inoculated with 10 µl of conidial suspensions after removing a small disk of shell. Controls were inoculated with sterile water only. In both cases the removed shell discs were placed back and sealed with parafilm. Ten fruits were inoculated with each fungal isolate. Inoculated and control nuts were incubated at $20 \pm 2^\circ\text{C}$ for 20 days in a moist chamber, then inspected for disease symptoms. Isolations from inoculated nuts were carried out as outline above. Experiments were repeated twice.

Three-year-old chestnut plants cv. Marrone di Susa were either potted and grown in a glasshouse or planted in a field about 20 km away from any chestnut stand. For the experiments on potted plants, young sprouts developed in the greenhouse were surface-sterilized with 2% sodium hypochlorite, wounded at their base with a sterile scalpel (wound size of about 1 mm²) and inoculated by dispensing on the wound 10 µl of conidial suspensions (2×10^7 conidia ml⁻¹). The inoculum was protected with moistened cotton and sealed with parafilm. Controls were inoculated as above with sterile water. Inoculations were carried out in early April 2008 on five sprouts of three plants per isolate. In September 2008 the fungus was tentatively re-isolated from the developed branches. To do so, branches were surface-disinfected, cut into transverse disks and plated as described above. In the field experiment, all flowers produced by five plants (5-10 each) were sprayed in spring 2009 with a conidial suspension (2×10^7 conidia ml⁻¹), and those produced by five additional plants (controls) were sprayed with sterile distilled water. At harvest, the nuts produced by these plants were for rot examined visually and used for re-isolations on MEA.

Microscopy. Three strains (NZ11, Rb7 and BvCo20) were incubated on MEA at 25°C for 10 days; conidiomata and conidia from each strain were measured under an Olympus BX40 light microscope (Olympus Corporation, Japan) at 400X magnification. Burrs were examined for the presence of the teleomorphic stage of the fungus with a Makroskop M420, stereo-microscope (Wild, Switzerland). Ascospores, asci, and ascospores were measured with the light microscope. Standard deviation and length:width ratio were determined for both conidia and ascospores. Some ascospores were cultured on MEA at 25°C to produce the anamorphic stage. Measurements of asci, ascospores and conidia are reported (see Results) as minimum and maximum values in parentheses, and ranges as intervals between the first and third quartile as suggested (Sogonov *et al.*, 2008). Arithmetic means, standard deviations and numbers of

measurements are provided in parentheses. Thus, measurements are expressed as length \times width = (min-)Q₁-Q₃(-max) \times (min-)Q₁-Q₃(-max) µm (mean1 \times mean2, SD1, SD2, n). Measures of microstructures are approximated to the nearest 0.5 µm.

Growth cardinal temperatures. Culture disks (6 mm in diameter) from an actively growing culture of a representative strain from Italy (VA24, Table 1) and from the strain NZ11 were individually transferred on MEA in Petri dishes (90 mm diameter) and incubated at 5, 10, 15, 20, 25, 30, $35 \pm 0.3^\circ\text{C}$. Five replicates per strain and temperature were plated. A data-logger unit monitored temperature values in each incubator during the whole experiment. After 4 and 7 days two orthogonal diameters of each colony were measured, corrected according to the size of the initial agar plug, and daily growth rates were recorded. This assay was repeated twice.

DNA extraction, PCR amplification, DNA sequencing and phylogenetic analysis. Phylogenetic analyses were carried out on a total of 15 fungal isolates, including 10 from Italy, three from Switzerland, one from France and one from New Zealand. All sequenced isolates were deposited at the Mycotheca Universitatis Taurinensis (MUT; http://web086.unito.it/cgi-bin/bioveg/documenti.pl/Show?_id=b522). See Table 1 for accession numbers). A holotypus was deposited at the Herbarium of the Dipartimento di Scienze della Vita e Biologia dei Sistemi, University of Turin (<http://www.bioveg.unito.it/italiano/erbario/index.html>) with accession number TO2545. Isolate MUT 401 (Rb7) is the ex-typus. DNA was extracted with the FTA Technology kit (Whatman, USA) following the manufacturer's instructions. Fungal mycelium actively growing on MEA was directly placed on the cards, from which a small portion was taken and added directly to the PCR mix. Regions of the rDNA repeat from the 3' end of the 18S gene to the 5' end of the 28S gene were amplified using primers ITS1/ITS4 (White *et al.*, 1990). Partial sequences of the EF1- α -coding gene were amplified with primers EF1-728F/EF1-1199R (Walker *et al.*, 2010). PCR was performed in a 25 µl mixture containing primers (0.4 µM each), 0.625 units of Go Taq polymerase (Promega stock solution 5 U µl⁻¹), 1X PCR buffer, dNTPs (0.4 mM) and template DNA. Cycling parameters consisted of an initial DNA denaturing step at 94°C for 2 min, followed by 35 amplification steps [94°C for 30 sec, 55°C (ITS1/4) or 58°C (EF1-728F/EF1-1199R and T1/T2) for 30 sec and 72°C for 1 min] and a final extension at 72°C for 10 min. Amplicons were either directly sequenced or cloned in pGEM-T (Promega, USA), and two independent clones per isolate were sequenced on both strands by BMR Genomics (<http://www.bmr-genomics.it/>). Publication-grade sequences were deposited in GenBank under accession Nos HM1142944-HM1142962, JQ898295-JQ898298

(ITS), and JQ791198-JQ791212 (EF1 α) (Table 1). Phylogenetic analyses were carried out using MEGA software (www.megasoftware.net). Neighbour-joining trees were constructed using Kimura's two-parameter model (Kimura, 1980) with 1000 bootstrap replicates, including sequences from other species retrieved from databases.

RESULTS

Frequency of isolation. Isolations from rotten chestnut nuts (Fig. 1A) collected in 2007 gave 53 to 83.3% positive recovery depending on the site of origin of the samples (see Materials and Methods), with an overall

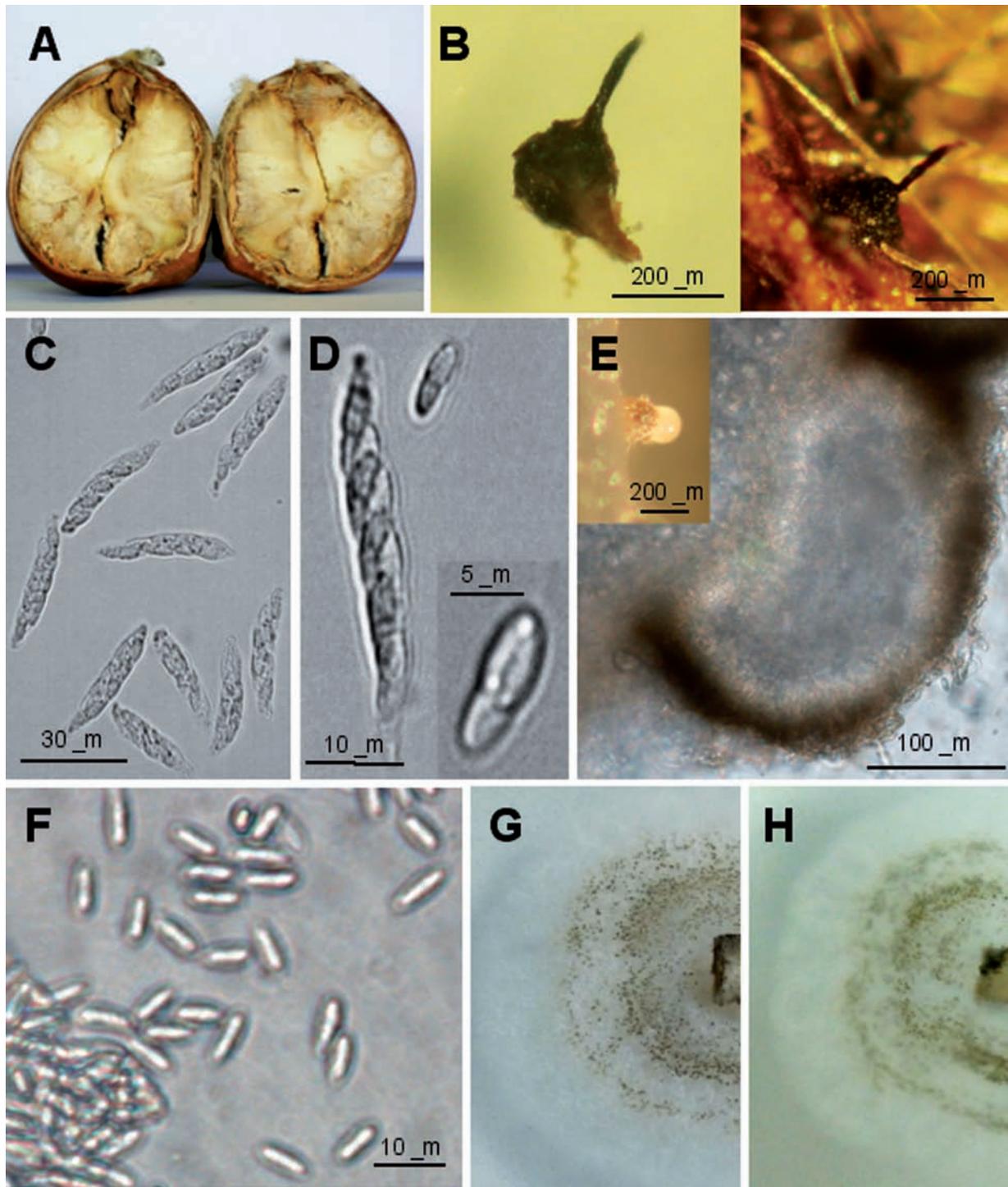


Fig. 1. Morphology of *Gnomoniopsis castanea* sp. nov. and disease symptoms on chestnut fruits. (A) Rotting of infected kernel. (B) Perithecia (left panel: isolated; right panel: inside a burr). (C) Asci with ascospores. (D) Ascospores. (E) Acervuli on MEA (inset: whole structure with drop containing conidia; main image: cryostatic section). (F) Conidia. (G) Front and (H) back side of the fungal colony actively growing on MEA.

Table 2. Disease incidence on chestnut nut samples collected in 2007-2011 in the Cuneo area (north-western Italy). Percentage of nuts infected by *Gnomoniopsis castanea* sp. nov. are given as a mean of percentage values obtained from a minimum of five chestnut stands in the area and 24 randomly collected nuts per orchard, \pm standard deviation.

Year	2007	2008	2009	2010	2011
Mean \pm SD	70.7 \pm 10.6	29.2 \pm 22.7	35.0 \pm 21.2	n.d.*	74.6 \pm 9.4

* not determined, because of the extremely poor yield.

mean of about 71%. More than 96% of the pieces from infected nut tissue yielded a fungus belonging to *Coelomyces*, while colonies of *Alternaria* spp., *Fusarium* spp., *Penicillium* spp. and *Trichoderma* spp. developed from the remaining nuts fragments. In the following years, the prevailing fungus was consistently isolated with a mean frequency ranging from 35.0% (2009) to 74.6% (2011) (Table 2). Isolations were also positive from the bark but not from the underlying xylem (Table 3).

The fungus was invariably able to reproduce the disease in all artificially inoculated chestnuts from which it was always re-isolated, thus fulfilling Koch's postulates.

By contrast, isolations from mock-inoculated control nuts were always unsuccessful. Conidial inoculation of flowers at pollination resulted in a percentage of diseased nuts hovering around 25%, whereas the inoculation of sprouts did not cause any visible symptoms on them and the ensuing branches. However, the pathogen was re-isolated from the bark all along the branches, 10-25 cm from the inoculation site, but never from branches that had been mock-inoculated at the sprout stage.

DNA sequence analysis. A phylogenetic analysis was carried out based on the sequence of the ITS region and

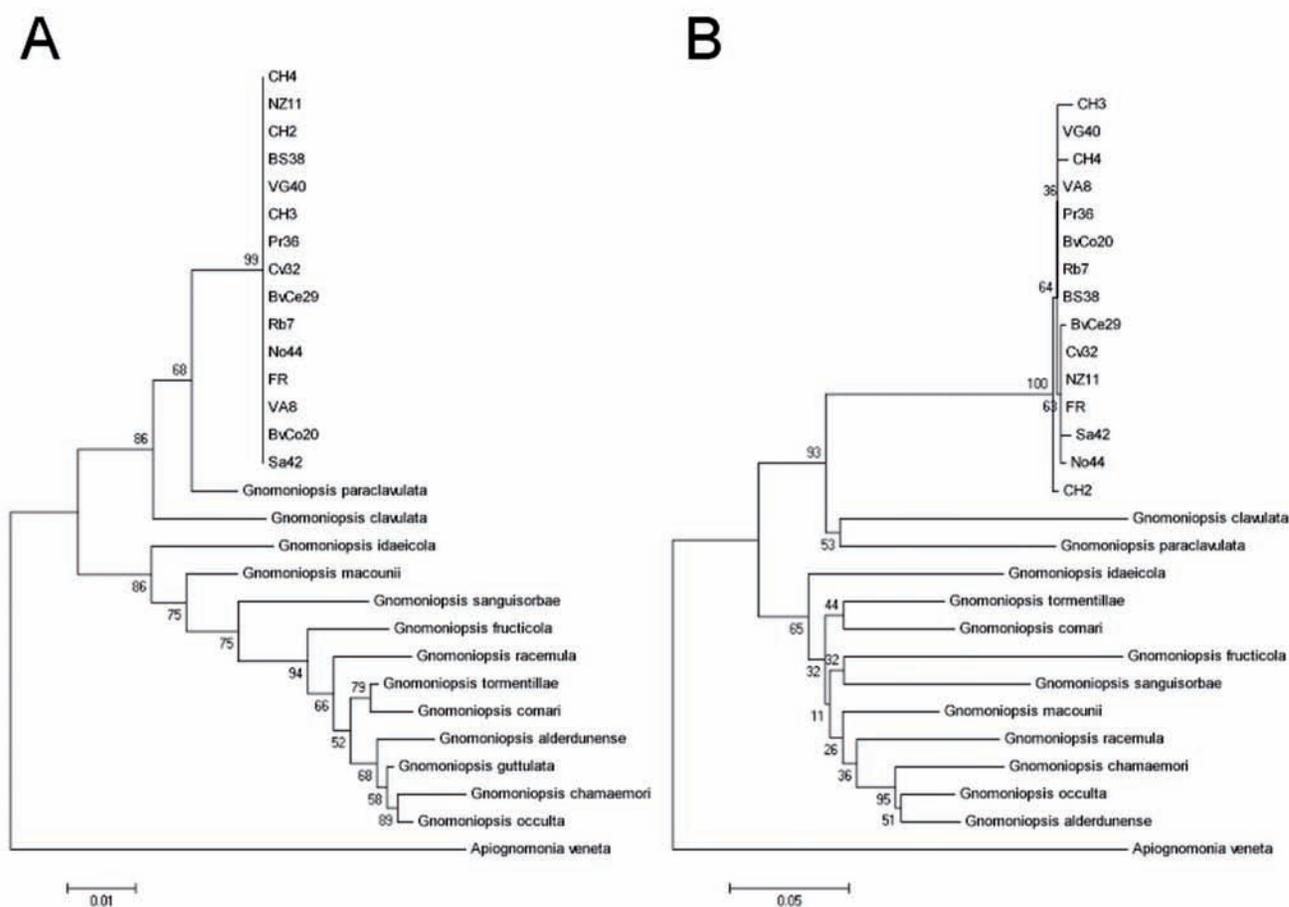


Fig. 2. Phylogeny of *Gnomoniopsis castanea* sp. nov. based on a multiple alignment of the ITS1-5.8S-ITS2 region of rDNA (A) and *EF1-α* locus (B). Neighbour-joining trees were constructed with Kimura's two-parameter model with 1000 bootstrap replicates. Edge length is indicated in terms of substitution rates per nucleotide, together with bootstrap % values. Sequences of fungal species different than *G. castanea* sp. nov. were retrieved from GenBank based on accession numbers reported by Walker and co-workers (Walker *et al.*, 2010).

Table 3. Frequency of isolation of *Gnomoniopsis castanea* sp. nov. from bark and xylem of young chestnut branches. Data are expressed as percentages of colonised specimens in samples collected between July 18th and August 28th 2008 in five chestnut stands located in the Cuneo area (north-western Italy). A minimum of 5 branches per age were randomly sampled in each stand.

Locality	Branch age (months)							
	6		12		24		36	
	bark	xylem	bark	xylem	bark	xylem	bark	xylem
Boves 1	100	0	95	0	100	0	100	0
Boves 2	100	0	95	0	88	0	100	0
Robilante 1	100	0	90	0	80	0	100	0
Robilante 2	100	0	97	0	43	0	39	0
Valdieri	100	0	70	0	75	0	100	0

Table 4. Size of conidia of three isolates of *Gnomoniopsis castanea* sp. nov.

Isolate	Diameter (μm)				Length (μm)			
	mean	min	max	SD	mean	min	max	SD
NZ11	3.355	2.44	4.88	0.779	7.32	6.10	9.76	0.970
Rb7	2.967	2.44	3.66	0.611	7.591	6.10	9.76	0.858
BvCo20	2.992	2.44	3.66	0.613	7.090	4.88	9.76	1.100

the *EF1- α* gene. Corresponding phylogenetic trees were generated with sequences from the 15 strains obtained in this study together with those of other *Gnomoniopsis* species from database. *Apiognomonina veneta* was used as an outgroup. The trees obtained by neighbour-joining analysis are shown in Fig. 2A and B for the ITS and *EF1- α* regions, respectively. BLAST comparison of the two sets of sequences for the 15 sequenced isolates gave very high similarity (>99%) within each set. For each *locus*, the closest sequences were those from *G. clavulata* or *G. paracavulata* (see Fig. 2A and B). The New Zealand isolate shared >99% identity with European isolates.

Taxonomy. *Gnomoniopsis castanea* G. Tamietti. MycoBank: MB 564970 (Fig. 1).

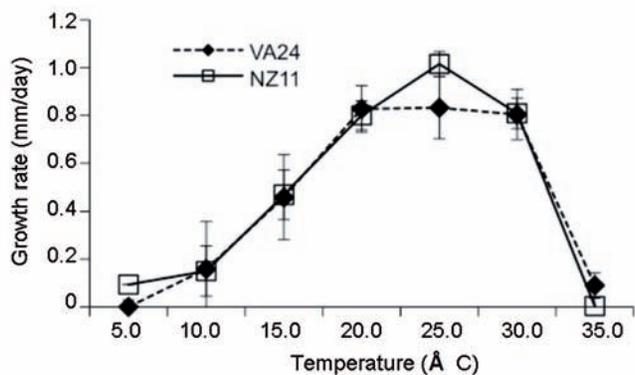


Fig. 3. Growth (mm/day) of isolates VA24 (Italy) and NZ11 (New Zealand) of *Gnomoniopsis castanea* sp. nov. at different temperatures. Data are means of 10 replicates. Bars represent standard deviations.

Holotypus. Italy, Robilante (CN), burr showing perithecia at the base of the spines; collected by G. Tamietti and S. Gentile, 2007. DBIOS Herbarium accession No. TO2545 (ex-typus: MUT 401).

Teleomorph. Abundant perithecia without stroma observed both inside and outside over-wintered burrs, at the base of the spines; some perithecia also present in rotten seeds. Perithecia immersed, sub-epidermal, black, oblate spheroidal when moist, 123-265 μm high \times 147-295 μm diameter (n=46), collapsed from the base when dry. Necks central, straight to slightly flexuous, 89-408 μm long, diameter 24.6-39.4 μm (n = 46) (Fig. 1B). Asci clavate or obclavate (29.3-34.2-41.5(-48.8) μm (4.9-7.3-6.1(-8.5) (mean 38.8 \times 6.8, SD1 4.1, SD2 0.9 μm , n = 85), apical ring 1.6-2.3 μm in diameter, containing eight uniseriate or irregularly multiseriate ascospores arranged mainly obliquely (Fig. 1C). Ascospores pyriform, l:w (2.7-3.6-4.0(-5.0) (mean = 3.8, SD 0.4, n = 58), two-celled, not constricted or slightly constricted at septum, septum located at (-38) 43-47 (49-)% (mean = 46, SD 0.05, n = 22) of ascospore length, ends blunt, rounded, each cell containing one big and several small guttules, or several indistinct guttules; appendages absent (Fig. 1D).

Anamorph cultures. Colonies on MEA attaining 90 mm after 9-11 days at 25°C, depending on the strain, flat, almost glabrous, overlaid by loose and short woolly-like mycelium, pale reddish grey with indistinct pale orange-brown patterns in the centre; margin diffuse (Fig. 1G); reverse of almost same colours as surface (Fig. 1H).

Acervuli profuse, closed, ellipsoid 244-268 \times 146-190 μm on MEA (n = 50), smaller (122-251 \times 85-171 μm) on

naturally infected fruits ($n = 20$) (Fig. 1E), bearing pale brown, one-celled, ovoid-oblong, biguttulate conidia, measuring $(2.4\text{--}2.4\text{--}3.7\text{--}(4.9)) \times (6.1\text{--}4.9\text{--}7.3\text{--}(9.8))$ (mean 3.0×7.2 μm , SD1 0.6, SD2 1.0, $n = 198$) μm , l:w (2.0–) 2.0–2.5(–.0) (mean = 2.41 μm , SD 0.4, $n=198$) μm , regardless of the substrate (Fig. 1F). The New Zealand strain has the same morphometric characters (Table 4). Macro- and micro-morphological characteristics of cultures obtained from ascospores are consistent with the above description.

Cardinal temperatures. The daily growth of isolates VA24 and NZ11 was very similar at most temperatures in the range between 5 and 35°C (Fig. 3). The optimum growth temperature was around 25°C with a daily growth of 0.83–1.01 mm, i.e. comparable to that recorded for other Italian isolates grown at this temperature only (data not shown).

Features. *Host and habitat:* *Castanea sativa* Mill., on overwintered burrs, in the bark of 6- to 36-month-old branches and in fruits.

Distribution: Italy, France, Switzerland, New Zealand (Smith and Agri, 2008). The actual geographical distribution is still undertermined.

Etymology: refers to the association with *Castanea sativa* Mill.

Additional material examined: Italy, Robilante (CN), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2007 (MUT 401); Italy, Valdieri (CN), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2008 (MUT 403); New Zealand, culture isolated from diseased *C. sativa* fruit by H. Smith, 2007 (MUT 411); Italy, Boves (CN), culture isolated from diseased *C. sativa* fruits by G. Tamietti, S. Gentile, 2007 (MUT 418); Italy, Boves (CN), culture isolated from symptomless branch of *C. sativa* by G. Tamietti and S. Gentile, 2008 (MUT 452); Italy, Ceva (CN), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2009 (MUT 455); Italy, Prarostino (TO), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2009 (MUT 802); Italy, Bassa Val Susa (TO), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2009 (MUT 806); Italy, Valgioie (TO), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2009 (MUT 807); Italy, Santena (TO), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2009 (MUT 808); Italy, Novara (NO), culture isolated from diseased *C. sativa* fruits by G. Tamietti and S. Gentile, 2009 (MUT 809); Switzerland, Biasca, culture isolated from diseased *C. sativa* fruits by M. Jermini, 2011 (MUT 810); Switzerland, Biasca, culture isolated from diseased *C. sativa* fruits by P. Gonthier and S. Gentile, 2011 (MUT 811); Switzerland, Cadenazzo, culture isolated from diseased *C. sativa* fruits by

P. Gonthier and S. Gentile, 2011 (MUT 814); France, Sisteron, culture isolated from diseased *C. sativa* fruits by P. Gonthier and S. Gentile, 2011 (MUT 815).

DISCUSSION

This study has shown that a fungal species for which the name *Gnomoniopsis castanea* sp. nov. is proposed, is a causal agent of chestnut nut rot in north-western Italy. Although other fungi were occasionally isolated from rotted chestnut nuts, *G. castanea* was by far the most frequently present. Phylogenetic analyses carried out on the rDNA ITS sequences, and parts of the EF1- α -coding regions, indicated that all strains sequenced obtained from nuts and the bark of young branches have a very low affinity with *Ciboria batschiana* and *Phomopsis* spp. (about 32 and 75% identity, respectively, for ITS sequences) and with *Gnomonia* spp., such as *G. orcispورا* (GenBank EU254789; 88% identity with the ITS sequence of isolate Rb7). Rather, in phylogenetic trees, the Italian isolates clearly grouped with French, Swiss and New Zealand isolates in a distinct clade, closely related to the *Gnomoniopsis* species *G. clavulata* and *G. paracavulata*. The morphological traits of the teleomorph agree with such taxonomic attribution (perithecia in groups, ascospores with a sub-median septum and without appendages). However, none of the *Gnomoniopsis* species described so far has phylogenetic, morphological and pathological characteristics overlapping those of *G. castanea*.

The causal agent of chestnut nut rot in New Zealand was proposed as the new species *Discula pascoe* (teleomorph: *Gnomonia pascoe*) (Smith and Agri, 2008). However, this species lacked a complete description at the morphological and, especially, the phylogenetic level and no holotypus was deposited in a public herbarium. These circumstances, which do not conform with a valid taxonomic identification (Seifert and Rossman, 2010), and the close phylogenetic relationships of the Italian fungal isolate with *Gnomoniopsis* species of good standing support the identification of *G. castanea* as a novel species. Furthermore, the comparative analysis of the European and New Zealand isolates at the morphological, physiological (i.e. growth temperatures) and phylogenetic levels did not detect differences that would justify their attribution to distinct species. The report of a *Gnomoniopsis* sp. as a chestnut nut rot agent in Australia seems to confirm the distribution of this disease in the southern hemisphere (Shuttleworth *et al.*, 2010). A *Gnomoniopsis* sp. has also been reported in association with a necrotic condition of chestnut leaves and galls induced by the gall wasp *Dryocosmus kuriphilus* (Magro *et al.*, 2010). In both cases, however, a detailed comparative analysis with the European isolates is needed to confirm species identity.

Gnomoniopsis castanea sp. nov. seems to be not only a nut pathogen, but also a symptomless chestnut endophyte. Indeed, during the artificial inoculation experiments, the fungus was invariably isolated 10-25 cm away from the inoculation site of inoculated, but not of control branches. It was also isolated with very high frequencies from the surface-sterilized bark of 6- to 36-month-old branches of naturally infected plants; one such isolate was included in our analyses (BvCe29; MUT 452). Neither lesions or fruiting bodies were ever found in naturally or artificially infected branches, at least under the environmental conditions of north-western Italy. Endophytism by *Gnomoniopsis* spp. has been reported before (Walker *et al.*, 2010).

G. castanea is an emergent disease agent in north-western Italy, as exemplified by the fact that in the last 5 years it was consistently associated with nut rot with high frequency. Additionally, it was isolated from rotten nuts from south-eastern France and southern Switzerland suggesting that the pathogen could be more widespread in Europe than initially thought. While the disease was successfully reproduced through the inoculation of flowers, further studies are needed to elucidate the biological cycle of the fungus, and to determine whether infection of nuts by the mycelium growing from symptomless branches may occur and be epidemiologically relevant.

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REFERENCES

- Barr M.E., 1978. The Diaporthales in North America with emphasis on *Gnomonia* and its segregates. *Mycologia Memoirs* **7**: 1-232.
- Baudry A., Robin C., 1996. Châtaignier: des plants, du bois, des fruits. Un point sur l'état sanitaire en France. *Arboriculture Fruitière* **499**: 21-28.
- Bolay A., 1971. Contribution à la connaissance de *Gnomonia comari* Karsten (syn. *G. fructicola* [Arnaud] Fall). Etude taxonomique, phytopathologique et recherches sur sa croissance *in vitro*. *Berichte der Schweizerischen Botanischen Gesellschaft* **81**: 398-482.
- Bounous G., Torello Marinoni D., 2005. Chestnut: botany, horticulture and utilization. In: Janick J. (ed.). Horticultural Reviews. John Wiley & Sons, Oxford, UK.
- Breisch H., 2008. Châtaignes: le point sur les pourritures. *Infos-Centre Technique Interprofessionnel des Fruits et Légumes* **240**: 30-33.
- Camici L., 1948. La "mummificazione delle castagne" da *Phomopsis viterbensis* sp. n. *Annali della Sperimentazione Agraria* **NS 2**: 557-566.
- Ciferri R., 1950. La "mummificazione" delle castagne da *Phomopsis endogena* (Speg.) nobis, n. comb. *Notiziario sulle Malattie delle Piante* **8**: 36-37.
- Cohen S.D., 2004. Endophytic selectivity of *Discula umbrinella* on *Quercus alba* and *Quercus rubra* characterized by infection, pathogenicity and mycelial compatibility. *European Journal of Plant Pathology* **110**: 713-721.
- Delatour C., Morelet M., 1979. La pourriture noire des glands. *Revue Forestière Française* **3**: 101-115.
- Kimura M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120.
- Maas J.L., 1998. Compendium of Strawberry Diseases. 2nd Ed. APS Press, St. Paul, MN., USA.
- Magro P., Speranza S., Stacchiotti M., Martignoni D., Papatratti B., 2010. *Gnomoniopsis* associated with necrosis of leaves and chestnut galls induced by *Dryocosmus kuriphilus*. *New Disease Reports* **21**: 15.
- Monod M., 1983. Monographie taxonomique des Gnomoniaceae (Ascomycètes de l'ordre des Diaporthales I). *Beiheft zur Sydowia* **9**: 1-315.
- Seifert K.A., Rossman A.Y., 2010. How to describe a new fungal species. *IMA Fungus* **1**: 10-16.
- Servazzi O., 1941. Appunti di Fitopatologia. VIII. Intorno alle muffe delle castagne. *Bollettino del Laboratorio Sperimentale e Regio Osservatorio di Fitopatologia di Torino* **17**: 1-5.
- Shuttleworth L., Guest D., Liew E., 2010. The biology and management of chestnut rot in south-eastern Australia. *28th Horticultural Congress, Lisbon, Portugal*.
- Sieber T.N., Jermini M., Conedera M., 2007. Effects of the harvest method on the infestation of chestnuts (*Castanea sativa*) by insects and moulds. *Journal of Phytopathology* **155**: 497-504.
- Smith H.C., Agri M., 2008. The life cycle, pathology and taxonomy of two different nut rot fungi in chestnut. *Australian Nutgrower* **22**: 11-15.
- Sogonov M.V., Castlebury L.A., Rossman A.Y., White J.F., 2007. The type species of *Apiognomonina*, *A. veneta*, with its *Discula* anamorph is distinct from *A. errabunda*. *Mycological Research* **111**: 693-709.
- Sogonov M.V., Castlebury L.A., Rossman A.Y., Mejía L.C., White J.F., 2008. Leaf-inhabiting genera of the Gnomoniaceae, Diaporthales. *Studies in Mycology* **62**: 1-77.
- Spegazzini C., 1879. La "mummificazione delle castagne" da *Phomopsis viterbensis* sp. n. *Nova addenda ad Mycologiam Venetam, Michelia* **1**: 453-487.
- Vettraino A.M., Paolacci A., Vannini A., 2005. Endophytism of *Sclerotinia pseudotuberosa*: PCR assay for specific detection in chestnut tissues. *Mycological Research* **109**: 96-102.
- Vogliano P., Bongini V., 1918. Sulla *Phoma endogena* parassita

- delle castagne. *Annali della Regia Academia d'Agricoltura di Torino* **60**: 3-12.
- Wadia K.D.R., Klinac D., McNeil D.L., 1999. Survey of chestnut nut-rot in New Zealand. *Proceedings 52nd New Zealand Plant Protection Conference, Auckland, New Zealand*: 136-140.
- Walker D.M., Castlebury L.A., Rossman A.Y., Sogonov M.V., White J.F., 2010. Systematics of genus *Gnomoniopsis* (Gnomoniaceae, Diaporthales) based on a three gene phylogeny, host associations and morphology. *Mycologia* **102**: 1479-1496.
- Washington W.S., Allen A.D., Dooley L.B., 1997. Preliminary studies on *Phomopsis castanea* and other organisms associated with healthy and rotted chestnut fruit in storage. *Australasian Plant Pathology* **26**: 37-43.
- Washington W.S., Stewart-Wade S., Hood V., 1999. *Phomopsis castanea*, a seed-borne endophyte in chestnut trees. *Australian Journal of Botany* **47**: 77-84.
- White T.J., Burns T., Lee S., Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds). *PCR Protocol: a Guide to Methods and Application*, pp. 315-322. Academic Press, New York, NY, USA.

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