Detection of Botryosphaeriaceae species within grapevine woody tissues by nested PCR, with particular emphasis on the *Neofusicoccum parvum*/*N. ribis* complex

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Abstract Several species of Botryosphaeriaceae and *Phaeomoniella chlamydospora* are common agents of grapevine decline worldwide. Currently, the use of culture independent PCR based techniques for detection of Botryosphaeriaceae within grapevine tissues has been limited to *Botryosphaeria dothidea*. In the present study, two Botryosphaeriaceae specific nested PCR assays were developed. One with a narrow target range, to detect *Neofusicoccum parvum* and the closely related species complex (*Neofusicoccum parvum*/*N. ribis sensu* Pavlic et al. Molecular Phylogenetics and Evolution 51:259–268, 2009) and another, with a wider range, to detect all 17 species of Botryosphaeriaceae which have been reported as potential wood pathogens of grapevine. The effectiveness of these assays was validated in vivo on naturally infected wood samples collected from standing vines and dormant grafted rooted cuttings commercialized in Italy by different nurseries in different years. All samples were also screened by means of a previously published nested PCR assay specific for *Phaeomoniella chlamydospora*. It was found that: 1) propagation material may play an important role as source of primary inoculum, not only of *Phaeomoniella chlamydospora*, as previously reported, but also for members of the Botryosphaeriaceae, among which *Neofusicoccum parvum*, *Botryosphaeria dothidea* and *Diplodia seriata* are the most common, and 2) multiple infections by different species belonging to Botryosphaeriaceae and/or *Phaeomoniella chlamydospora* occur frequently both in standing vines and propagation material. This last finding supports the hypothesis that at least some of the non-specific symptoms of grapevine decline may be due to the presence of different pathogens within host tissues.

Keywords Grapevine decline · Multiple infections · *Neofusicoccum parvum* · Nursery propagating material · Sources of inoculum

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Introduction

Among the fungi which are known to cause trunk diseases and grapevine decline (Rolshausen et al. 2010), increasing importance has been given to *Phaeomoniella chlamydospora* (W. Gams, P. Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams (Crous and Gams 2000) and to species in Botryosphaeriaceae Theiss. & H. Syd. (Crous et al. 2006). *Phaeomoniella chlamydospora* alone or together with species of

Phaeoacremonium is the causal agent of a complex disease that is characterized by three different syndromes: Brown wood streaking (mostly affecting rooted cuttings), Petri disease and Grapevine leaf stripe disease (previously known as "young esca") (Surico 2009). The Botryosphaeriaceae comprise cosmopolitan Ascomycetes occurring worldwide as saprophytes, endophytes or pathogens on a wide range of gymnosperms, monocotyledons and dicotyledons, including the grape *Vitis vinifera* L. (Crous et al. 2006; van Niekerk et al. 2006). To date, 17 potentially phytopathogenic species of this family have been associated with cases of dieback of grapevines, bud mortality, brown streaking or necrosis of the wood, grape rot, delayed bud burst (Luque et al. 2005; van Niekerk et al. 2006; Damm et al. 2007; Martin and Cobos 2007; Carlucci et al. 2009; Aroca et al. 2010) as well as different leaf symptoms (Cristinzio 1978; Larignon et al. 2001). Pathogenicity tests have shown that *Neofusicoccum parvum* is one of the most virulent Botryosphaeriaceae species on grapevine worldwide (Phillips 2002; van Niekerk et al. 2004; Úrbez-Torres and Gubler 2009).

Together with *Neofusicoccum ribis*, *N. parvum* was considered to be part of the *Botryosphaeria dothidea* complex (Slippers et al. 2004). Currently, these two *Neofusicoccum* species, together with three cryptic species isolated from *Syzygium cordatum* in South Africa, are regarded as forming a unique group, named the *N. parvum/N. ribis* complex (Pavlic et al. 2009). *Neofusicoccum parvum* has been frequently associated with brown streaking and necrosis of grapevine wood (Phillips 2002; van Niekerk et al. 2006) whereas *N. ribis* has been isolated only occasionally from grape bunch rots (Pascoe 1998) as well as from discoloured wood (Crous et al. 2000). Nevertheless, the results of pathogenicity tests on grapevine carried out with a strain isolated from *Ribes* sp. indirectly suggest that this species could also be an important wood pathogen of *Vitis* sp. (van Niekerk et al. 2004).

The problem of the early diagnosis of *P. chlamydospora* has been addressed by the development of several PCR based protocols which have been shown to be efficient and reliable to detect the fungus from grapevine tissues, water, soil and grafting tools. Surveys carried out with these tools have shown that vegetative propagation materials are frequently infected by *P. chlamydospora* (Retief et al. 2006;

Edwards et al. 2007). On the other hand, with one exception (Romanazzi et al. 2009), diagnosis of the various species in Botryosphaeriaceae which may infect grapevine tissues, is currently based on their isolation on agar media followed by characterization by PCR fingerprinting (Alves et al. 2007), dot blot hybridization (Martos et al. 2009) or sequence analysis of one or more DNA regions (van Niekerk et al. 2004; Úrbez-Torres et al. 2006). It is well documented that these procedures, especially when carried out using multiple gene sequence data, are extremely reliable for identification. Nevertheless, they are time consuming and their sensitivity is limited by the fact that a minimum density of the target organism within the sample is required for isolation.

In this study, two independent nested PCR assays were developed based on sequences of the ITS1-5,8S-ITS2 rDNA (ITS rDNA) region (White et al. 1990) of species in Botryosphaeriaceae. The first assay (NprcA/NprcB assay) aimed at detecting the fungi belonging to the *N. parvum/N. ribis* complex *sensu* Pavlic et al. (2009) whereas the second (BoitsA/BoitsB assay) aimed at specifically amplifying the DNA of all the species in Botryosphaeriaceae, including *N. parvum* and *N. ribis*, which have been associated with wood diseases of grapevine. By means of these two molecular tools, as well as one (Pch1/Pch2 assay) specific for *P. chlamydospora* (Tegli et al. 2000; Edwards et al. 2007), we evaluated the relative incidence of these species within the woody tissues of standing vines and in nursery propagating material currently commercialized in Italy.

Materials and methods

Fungal and bacterial strains

To assess the in vitro specificity of the primer sets developed or employed in this study, 81 reference strains of fungi and bacteria (Table 1) and 43 strains belonging to Botryosphaeriaceae, isolated and characterized during this study (Table 2), were tested. All strains, except those of *Dothiorella iberica, Dothiorella sarmentorum, Diplodia corticola* and *Neofusicoccum ribis*, were isolated from grapevine wood in different countries. All fungi were grown in 30 ml 2% potato dextrose broth (Difco laboratories Inc., Detroit,

Table 1 Fungal and bacterial reference strains and fungal isolates used in this study to verify the specificity of the primer sets BoitsA/BoitsB (Botryosphaeriaceae specific), NprcA/NprcB (*N. parvum*/*N. ribis* complex specific) and Pch1/Pch2 (*P. chlamydospora* specific)

Identity	Host and geographical origin	Collector	Amplification with each primer set		
			BoitsA/BoitsB (this study)	ParA/ParB (this study)	Pch1/Pch2[a]
Reference strains					
Botryosphaeria dothidea					
UCD2190Mo	Grapevine; USA	Úrbez-Torres J. R.	+[b]	–	–
UCD2255Mo	Grapevine; USA	Úrbez-Torres J. R.	+	–	–
UCD2191	Grapevine; USA	Úrbez-Torres J. R.	+	–	–
PVFi-Bd1	Grapevine; Italy	Rapetti S.	+	–	–
PVFi-Bd2	Grapevine; Italy	Natalini G.	+	–	–
Diplodia mutila					
M226	Grapevine; New Zealand	Jaspers M.	+	–	–
N1351	Grapevine; New Zealand	Jaspers M.	+	–	–
CSU-07-WP-TS23	Grapevine; Australia	Savocchia S.	+	–	–
CSU-07-WP-CV5	Grapevine; Australia	Savocchia S.	+	–	–
UCD1953SB	Grapevine; USA	Úrbez-Torres J.R.	+	–	–
UCD2165OR	Grapevine; USA	Úrbez-Torres J.R.	+	–	–
Diplodia seriata					
B121	Grapevine; New Zealand	Jaspers M.	+	–	–
N144	Grapevine; New Zealand	Jaspers M.	+	–	–
CSU-07-WP-DO4	Grapevine; Australia	Savocchia S.	+	–	–
CSU-07-WP-J10	Grapevine; Australia	Savocchia S.	+	–	–
UCD244Ma	Grapevine; USA	Úrbez-Torres J.R.	+	–	–
UCD352Mo	Grapevine; USA	Úrbez-Torres J.R.	+	–	–
UCD2161	Grapevine; USA	Úrbez-Torres J.R.	+	–	–
PVFi-Ds1	Grapevine; Italy	Natalini G.	+	–	–
Diplodia corticola					
CBS112077	*Quercus suber*; Spain	Luque J.	+	–	–
CBS112076	*Quercus suber*; Spain	Luque J.	+	–	–
Dothiorella iberica					
CBS 115035	Oak; Spain	Luque J.	+	–	–
CBS 115041	Oak; Spain	Luque J.	+	–	–
Dothiorella sarmentorum					
CBS 120.41	Pear; USA	Wollenweber H.W.	+	–	–
Dothiorella viticola 573					
(anamorph of *S. viticola*)	Grapevine; Spain	Luque J.	+	–	–
Lasiodiplodia theobromae					
CBS 124060	Grapevine; Italy	Burruano S.	+	–	–
UCD205Co	Grapevine; USA	Úrbez-Torres J. R.	+	–	–
UCD2193	Grapevine; USA	Úrbez-Torres J. R.	+	–	–
UCD1014BC	Grapevine; Mexico	Úrbez-Torres J. R.	+	–	–
Lasiodiplodia plurivora					
CBS 121103	Grapevine; South Africa	Halleen F.	+	–	–

Table 1 (continued)

Identity	Host and geographical origin	Collector	Amplification with each primer set		
			BoitsA/BoitsB (this study)	ParA/ParB (this study)	Pch1/Pch2[a]
Neofusicoccum australe					
Kat-1	Grapevine; New Zealand	Jaspers M.	+	–	–
Mel-1	Grapevine New Zealand	Jaspers M.	+	–	–
CSU-07-WP-FF24	Grapevine; Australia	Savocchia S.	+	–	–
CSU-07-WP-M2	Grapevine; Australia	Savocchia S.	+	–	–
Neofusicoccum luteum					
N-1	Grapevine; New Zealand	Jaspers M.	+	–	–
M(8)4	Grapevine; New Zealand	Jaspers M.	+	–	–
UCD2057Te	Grapevine; USA	Úrbez-Torres J. R.	+	–	–
UCD2090Te	Grapevine; USA	Úrbez-Torres J. R.	+	–	–
Neofusicoccum parvum					
CBS 117923	Guava; Venezuela	Cedeno L.	+	+	–
G22a3	Grapevine; New Zealand	Jaspers M.	+	+	–
B2141	Grapevine; New Zealand	Jaspers M.	+	+	–
UCD2205Mo	Grapevine; USA	Úrbez-Torres J. R.	+	+	–
UCD2280Mo	Grapevine; USA	Úrbez-Torres J. R.	+	+	–
UCD2194	Grapevine; USA	Úrbez-Torres J. R.	+	+	–
Neofusicoccum ribis					
CBS 115475 (CMW7772)	*Ribes* sp. USA	B. Slippers & G. Hudler	+	+	–
Neofusicoccum viticlavatum					
CBS 112878	Grapevine; South Africa	Hallen F.	+	–	–
Neofusicoccum vitifusiforme					
CBS110887	Grapevine; South Africa	van Niekerk J.	+	–	–
Phaeobotryosphaeria porosa					
CBS110574	Grapevine; South Africa	van Niekerk J.	+	–	–
Spencermartinsia viticola					
CBS 117009	Grapevine; Spain	Luque J.	+	–	–
Phaeoacremonium aleophilum					
PVFi-Pal72	Grapevine; Italy	Bertelli E.	–	–	–
PVFi-Pal80	Grapevine; Italy	Bertelli E.	–	–	–
PVFi-Pal86	Grapevine; Italy	Mugnai L.	–	–	–
PVFi-Pal94	Grapevine; USA	Van der Water L.	–	–	–
PVFi-Pal95	Grapevine; USA	Mugnai L.	–	–	–
CBS 631.94	Grapevine; Italy	Mugnai L.	–	–	–
Phaeoacremonium angustius					
PVFi-Pang29	Grapevine; Italy	Bertelli E.	–	–	–
CBS 249.95	Grapevine; USA	Larignon P.	–	–	–
Phaeoacremonium inflatipes					
CBS 391.71	Oak; USA	Halliwell L. S.	–	–	–
CBS 100818	Olive; Italy	Frisullo S.	–	–	–
Phaeoacremonium rubrigenum					
CBS 498.94	Human; USA	Kwong-Chung K. J.	–	–	–

Table 1 (continued)

Identity	Host and geographical origin	Collector	Amplification with each primer set		
			BoitsA/BoitsB (this study)	ParA/ParB (this study)	Pch1/Pch2[a]
Phaeomoniella chlamydospora					
PVFi-Pch36	Grapevine; Italy	Bertelli E.	–	–	+
PVFi-Pch44	Grapevine; Italy	Mugnai L.	–	–	+
CBS 229.95	Grapevine; Italy	Mugnai L.	–	–	+
PVFi-Pch128	Grapevine; Italy	Bertelli E.	–	–	+
PVFi-Pch132	Grapevine; Italy	Mugnai L.	–	–	+
Bionectria ocralucea					
PVFi-Bn1	Grapevine; Italy	Spagnolo A.	–	–	–
PVFi-Bn2	Grapevine; Italy	Spagnolo A.	–	–	–
Cylindrocarpon destructans					
Cyl 204.03	Grapevine; New Zealand	Jaspers M.	–	–	–
Cyl 207.03	Grapevine; New Zealand	Jaspers M.	–	–	–
Phomopsis viticola					
PVFi-ph1	Grapevine; Italy	Mugnai L.	–	–	–
PVFi-ph3	Grapevine; Italy	Mugnai L.	–	–	–
Botrytis cinerea					
PVFi-Bc3	Grapevine; Italy	Mugnai L..	–	–	–
Aspergillus sp.					
PVFi-As1	Grapevine; Italy	Mugnai L.	–	–	–
Alternaria sp.					
PVFi-Al1	Grapevine; Italy	Mugnai L.	–	–	–
Penicillium sp.					
PVFi-Pn5	Grapevine; Italy	Mugnai L.	–	–	–
Eutypa lata					
PVFi-El04	Grapevine; Croatia	Peduto F.	–	–	–
PVFi-Ell2	Grapevine; Italy	Mugnai L.	–	–	–
Fomitiporia mediterranea					
PVFi-Fom200.03	Grapevine; Italy	Mugnai L.	–	–	–
PVFi-Fom201.03	Grapevine; Italy	Marchi G.	–	–	–
Agrobacterium vitis					
ICMP 12847	Grapevine; Japan	Sawada H.	–	–	–
PVFi-Av5	Grapevine; New Zealand	Peduto F.	–	–	–

[a] Tegli et al. (2000)

[b] +and—indicates the presence or the absence of the expected diagnostic amplicon

MI, USA) at 25°C in the dark on a rotary shaker (100 rpm) for 7–14 days. Approximately 200 mg of actively growing mycelium were collected aseptically for DNA extraction. *Agrobacterium vitis* strains were grown for 48 h at 26°C on Nutrient agar (Difco laboratories Inc., Detroit, MI, USA). Single colonies were subjected to DNA extraction.

Plant material

The three molecular assays were tested in vivo on two different types of naturally infected samples: i) chips from the trunk and arms of standing vines or the rootstock trunk of dormant grafted rooted cuttings [chip samples]; ii) pooled samples of the rootstock

Table 2 Verification of the specificity of the primer sets BoitsA/BoitsB (Botryosphaeriaceae specific), NprcA/NprcB (*N. parvum*/*N. ribis* complex specific) and Pch1/Pch2 (*P. chlamydospora* specific) using the Botryosphaeriaceae isolates recovered in this study

Identity	Host	Amplification with each primer set		
		BoitsA/BoitsB (this study)	ParA/ParB (this study)	Pch1/Pch2[a]
B. dothidea				
PVFi-Bd3–5	Standing vine	+	–	–
D. seriata				
PVFi-Ds2–11	Standing vine	+	–	–
N. parvum				
PVFi-Np2–10	Standing vine	+	+	–
PVFi-Np11–13	DGRC 2007 (A[c])	+	+	–
PVFi-Np14–31	Standing vine	+	+	–

[a] Tegli et al. (2000)

[b] +and—indicates the presence or the absence of the expected diagnostic amplicon

[c] the capital letter indicates the nursery of provenience of the sampled DGRC

trunks of dormant grafted rooted cuttings or canes of *Vitis vinifera* subsp. *sylvestris* [pooled samples].

Chip samples

Five standing vines (cv. Sauvignon blanc) were uprooted in 2007 from a twelve year old commercial vineyard in the province of Florence (Italy). In the same year five Dormant Grafted Rooted Cuttings (DGRC 2007) were chosen randomly from each of three lots (different combinations of cultivars and/or rootstocks) produced by nursery B (central Italy) and from one lot produced by nursery E (France). Vines and DGRC 2007 were washed with tap water, cross sectioned, surface disinfected as described by Retief et al. (2006), and the bark was removed with a sterile scalpel. The sections of the trunk and arms (standing vines) or rootstock trunk of the DGRC 2007 were then cut longitudinally and, if present, the position and the extent of the wood discolouration noted. Fifty-four samples were taken from discoloured wood of standing vines and 45 from DGRC 2007, of which 24 were from discoloured and 21 were from non-discoloured wood. All samples were processed immediately for DNA extraction and fungal isolation.

Pooled samples

Thirteen lots of dormant grafted rooted cuttings (different combinations of cultivars and/or rootstocks)

ready to be commercialized in Italy by four national nurseries [A and B, (central Italy); C and D (northern Italy)] and one French nursery (E), were inspected in 2008 (DGRC 2008). From each lot of material, a minimum of five DGRC 2008 were chosen randomly, the scions and the roots removed with sterile pruning shears and the rootstock trunk was surface disinfected as described above. Each of the five trunks was cut into segments approximately 5–7 cm long and five segments, one from each trunk, were sub-sampled. After bark removal with a sterile scalpel, the resulting woody tissues comprised the pooled sample to be analysed (average weight 13.7±5.8 g). Based on the size of each sampled lot, the sampling procedure was replicated from one to nine times, resulting in a total of 50 pooled samples. Ten canes were taken from two wild *Vitis vinifera* subsp. *sylvestris* vines located in the World Wildlife Fund (WWF) Conservation Area of the Burano Lake in Tuscany (Grosseto, Italy) and processed as described above. All samples were immediately processed for DNA extraction.

Isolation and characterization of Botryosphaeriaceae species and *P. chlamydospora* from plant material

From 10 to 36 wood chips (2–5 mm) per sample of standing vines and DGRC 2007 were placed on 2% potato dextrose agar (PDA, Difco laboratories Inc., Detroit, MI, USA) amended with 0.015% streptomy-

cin sulphate (Sigma-Aldrich Corporation, Saint Louis, MO, USA) and incubated at 25°C in the dark for up to 3 weeks. Isolates were transferred on PDA and, after microscopic examination of fruiting structures and conidia, up to four monoconidial cultures per sample were obtained according to Tuite (1969) from isolates that could be ascribed to Botryosphaeriaceae (Phillips 2002). More than one isolate (up to four) per sample was chosen when different morphometric or colony features were noted. Identification to species level was done by sequence analysis of the ITS rDNA according to Úrbez-Torres et al. (2006) with minor modifications. Isolates that could be ascribed to species belonging to the *N. parvum/N. ribis* complex, were further characterized by sequencing parts of the β-tubulin (BT) (Úrbez-Torres et al. 2006) and translation elongation factor 1-alpha (EF1-α) (van Niekerk et al. 2004) genes, and subsequent phylogenetic analysis. *P. chlamydospora* isolates were morphologically identified according to Crous and Gams (2000).

DNA extraction

Fungal mycelia and wood chips (average weight 98± 20 mg) from standing vines and DGRC 2007 were ground with sterile inert sand and liquid nitrogen in a sterile ceramic mortar with the addition of 800 μl of the AP1 buffer of the DNeasy Plant Minikit (Qiagen N.V., Venlo, Netherlands). Pooled wood samples (DGRC 2008) were homogenized for 3 min in 100 ml of sterile phosphate buffer (0.05 M, pH 7.2) using a refrigerated Waring blendor (Waring Products, Torrington, CT, USA). One ml of homogenate was centrifuged for 5 min at $1000 \times g$ and the supernatant was transferred to a new sterile tube and centrifuged again for 10 min at $9200 \times g$ and the supernatant discarded. In all cases further steps of DNA extraction were carried out with the DNeasy Plant Minikit according to the manufacturer's instructions. To ensure that the DNA had been successfully extracted and that no PCR inhibitors were present, the purified DNA was amplified with the universal primer set ITS5/ITS4 (White et al. 1990). DNA was stored at -80°C until further use.

Primer design and set up of two nested PCR assays

The ITS rDNA sequences of 16 Botryosphaeriaceae species reported from grapevine wood were retrieved from the International Nucleotide Sequence Database (INSD; http://www.insdc.org/). Nucleotide sequences were aligned with ClustalX (http://www.clustal.org/) and adjusted manually when necessary. Two primer sets were designed within the regions flanked by the universal primers ITS5 and ITS4 (White et al. 1990): NprcA (5'- AACTCCAGTCAGTGAACT -3') and NprcB (5'- CCGAGGTCAACCTTGAGAAAT -3') to specifically amplify a region of 372 bp in the species belonging to the *N. parvum/N. ribis* complex (Pavlic et al. 2009); BoitsA (5'- GACCATCA AACTCCAGTCAG -3') and BoitsB (5'- AAAGTTC AGAAGGTTCGTCCGG -3') to specifically amplify a region varying from 359 to 365 bp in the Botryosphaeriaceae species included in this study. Their theoretical specificity was tested *in silico* using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi).

As the quantity of target DNA in the naturally infected samples was expected to be low, nested PCR specific assays were set up. PCR reagents were purchased from Fermentas International Inc., (Burlington, Ontario, Canada) and primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All PCR reactions contained the following reagents in a final volume of 25 μl: 0.5 mM $MgCl_2$, 0.2 mM dNTP's mix, 1x DreamTaq™ Buffer ($MgCl_2$ 2 mM + KCl + $(NH_4)_2SO_4$), 0.2 μM of each primer, 1.25 U of DreamTaq™ DNA Polymerase and 2 μl of DNA template. Reaction mixtures were assembled on ice and performed in a preheated thermal cycler (Bio-Rad laboratories, Inc., Hercules, CA, USA). The first round of the nested PCR, in common to both assays, was carried out with the universal primer set ITS5/ITS4 (White et al. 1990). Thermal cycling conditions for this amplification were 3 min at 94°C followed by 30 cycles, each of which consisted of: 30 s at 94°C, 30 s at 50°C, 30 s at 72°C, and a final extension step of 7 min at 72°C. The resulting PCR products as well as the negative controls of PCR were diluted 1:100 with double distilled sterile water and 2 μl were used as template in the second round of PCR, carried out with the primer set NprcA/NprcB for the *N. parvum/N. ribis* complex specific assay or with the primer set BoitsA/BoitsB for the Botryosphaeriaceae specific assay. Thermal cycling conditions for the amplification with the primer set NprcA/NprcB were: 5 min at 94°C followed by 40 cycles, each of which consisted of: 45 s at 94°C, 30 s at 61°C, 30 s at 72°C, and a final

extension step of 7 min at 72°C. Thermal cycling conditions for the amplification with the primer set BoitsA/BoitsB were the same as for NprcA/NprcB except that 35 cycles instead of 40 were used. First round PCR products were also amplified with the primer set Pch1/Pch2 reported as specific for *P. chlamydospora* (Tegli et al. 2000), according to the PCR conditions described by Edwards et al. (2007).

All amplified PCR products were resolved by electrophoresis at 100 Vcm^{-1} in 2% agarose (IBI-Shelton Scientific, Peosta, IA, USA) gels in 1x Tris-borate-EDTA buffer stained with ethidium bromide ($0.5 \mu gml^{-1}$), and visualized under a UV transluminator (Bio-Rad laboratories, Inc., Hercules, CA, USA). Fragment size was estimated with Mass Ruler DNA Ladder low range (Fermentas International Inc., Burlington, Ontario, Canada). To reduce the risk of cross-contamination, all manipulations (DNA extraction, PCR reactions set up and electrophoresis) were performed in separate rooms with dedicated tools. Filter pipette tips were always used.

In vitro sensitivity of the nested PCR assays

The genomic DNA concentration of *N. parvum* PVFi-Np9, *Spencermartinsia viticola* CBS117009 and *P. chlamydospora* PVFi-Pch36 (Tables 1 and 2) was measured using a DyNA Quant 200 fluorometer (Hoefer Inc., Holliston, MA, USA) and 1:10 serial dilutions were made with double distilled sterile water. The sensitivity of the nested PCR was estimated at the maximal dilution titre by using the NprcA/NprcB, BoitsA/BoitsB and Pch1/Pch2 primer sets, respectively, both in single step and nested PCR.

Sequencing and phylogenetic analyses

PCR products were purified with the EXOSAP mix (USB Europe GmbH, Staufen, Germany) in accordance with manufacturer's instructions and sequenced on a ABI prism 310 CE system (Applied Biosystem, Foster City, CA, USA), using the ABI prism Big Dye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystem, Foster City, CA, USA). Chromatograms, including sense and antisense sequences, were edited and assembled manually to obtain single consensus sequences. Primer sequences were removed and identity searches were performed on the INSD database.

Bayesian analyses were performed for each of the ITS-rDNA, BT and EF1-α data sets and, after checking their congruence by means of the partition homogeneity test as implemented in PAUP* v.4.0b10 (Swofford 2002), on the concatenated ITS-rDNA and EF1-α data sets, including at least one reference sequence of each of the species in Botryosphaeriaceae that have been reported from grapevine wood. When needed, the reference sequences of Botryosphaeriaceae species isolated from other hosts were also considered. Sequences were aligned with ClustalW using the alignment option in Mega 4.1 (Tamura et al. 2007) and the analyses were performed with MrBayes v 3.1.2 (Huelsenbeck and Ronquist 2001) using *B. dothidea* CMW8000 (acc. numbers: AY236949, AY236927, AY236898) as the outgroup. The MCMC analysis ran for 1×10^6 generations, with a sample frequency of 100 and a burn-in of 2500. Three independent runs were performed for each dataset to check the congruence of the results. The resulting consensus trees were edited with Figtree v 1.2.1 (http://tree.bio.ed.ac.uk/).

The specificity in vivo of the BoitsA/BoitsB and NprcA/NprcB assays and the frequency of samples infected by different species from Botryosphaeriaceae was evaluated using DNA samples that tested positive to both independent assays. To determine the identity of the DNA template, 42 BoitsA/BoitsB amplicons (ten from standing vines, ten from DGRC 2007, 20 from DGRC 2008 and two from *V. vinifera* subsp. *sylvestris*) and of 15 NprcA/NprcB amplicons (four from standing vines, four from DGRC 2007, five from DGRC 2008 and two from *V. vinifera* subsp. *sylvestris*), were sequenced and included in a phylogenetic analysis as described above using *Guignardia bidwelli* CBS111145 (acc. number EU683672) as the outgroup.

Statistical analysis

Observed frequencies of positive and negative samples for each of the three independent nested PCR assays or for fungal isolation, were compared as unordered R×C contingency tables using the two-tailed "Fisher-Freeman-Halton" exact test as implemented by StatXact (CYTEL Software Corporation, Cambridge, MA, USA). Differences between compared groups were considered statistically significant at P≤0.05.

Results

Isolation and characterization of the Botryosphaeriaceae and *P. chlamydospora*

Isolations from discoloured wood from standing vines indicated the presence of Botryosphaeriaceae in 28 and *P. chlamydospora* in 10 of the 54 samples analyzed (Fig. 1a). Although as a whole Botryosphaeriaceae were present in all and *P. chlamydospora* in four out of five of the sampled vines, only three

Fig. 1 Detection of *N. parvum/N. ribis*, species in Botryosphaeriaceae and *P. chlamydospora* in naturally infected woody tissue samples (chip samples) by means of three independent nested-PCR specific assays (M) and direct fungal isolation (I). For standing vines **a**, samples subjected to analysis were taken only from discoloured wood, whereas for DGRC 2007 **b** both from discoloured and non discoloured wood. An asterisk above a pair of vertical bars indicates a statistically significant difference (P≤0.05) in the frequencies of positive samples between detection methodologies, based on a "Fisher-Freeman-Halton" exact test analysis

samples were co-infected with both taxonomical units (data not shown). With the same analysis on discoloured and non-discoloured wood from DGRC 2007, Botryosphaeriaceae-like morphotypes were isolated from only three out 24 of the discoloured wood samples, while *P. chlamydospora*-like morphotypes could never be isolated (Fig. 1b). As a whole two out of 20 DGRC 2007 were found to be infected by Botryosphaeriaceae (data not shown). Based on the sequence analysis of the ITS rDNA, 40 Botryosphaeriaceae isolates were obtained from 28 discoloured wood samples of standing vines, of which 27 isolates could be ascribed to *N. parvum*, ten to *D. seriata* and three to *B. dothidea* (Table 2). Different species of Botryosphaeriaceae were isolated from the same plant but never together from the same sample of discoloured wood (data not shown). In the case of DGRC 2007, all three Botryosphaeriaceae isolates analyzed were ascribed to *N. parvum* (Table 2). BT and EF1-α individual gene sequences, always confirmed the preliminary identification of all the *N. parvum* isolates. As a whole, two groups of sequences resulted for each one of the ITS (acc. numbers GU187981–GU188010) and EF 1-α (acc. numbers GU188011–GU188040) sequence analyses whereas BT sequences were identical for all the isolates (data not shown). Results of the partition homogeneity test indicated that there was no significant incongruence (P=0.133) between the EF 1-α and ITS rDNA data sets. Phylogenetic analysis of combined sequences revealed three distinct phylogenetic groups of *N. parvum* (Fig. 2). Two out of these groups were present both in standing vines and DGRC 2007 (Table 2, Fig. 2). Although at least two of the three phylogenetic groups could be isolated from the same plant, they were never isolated together from the same wood discolouration (data not shown).
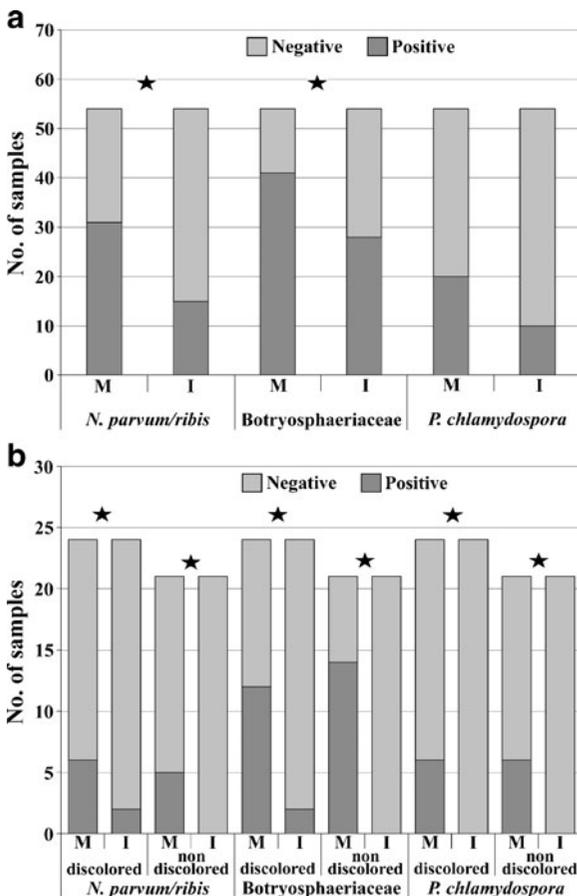
Evaluation of the PCR assays in vitro

Both the primers NprcA and NprcB showed a theoretical identity equal to 100% toward their respective target regions, whereas the identity values between the primers BoitsA and BoitsB and their respective target regions were variable. All the DNAs extracted from reference strains or fungi isolated during this study were successfully amplified with the universal primer set ITS5/ITS4. The primer sets NprcA/NprcB (*N. parvum/N. ribis* complex specific),
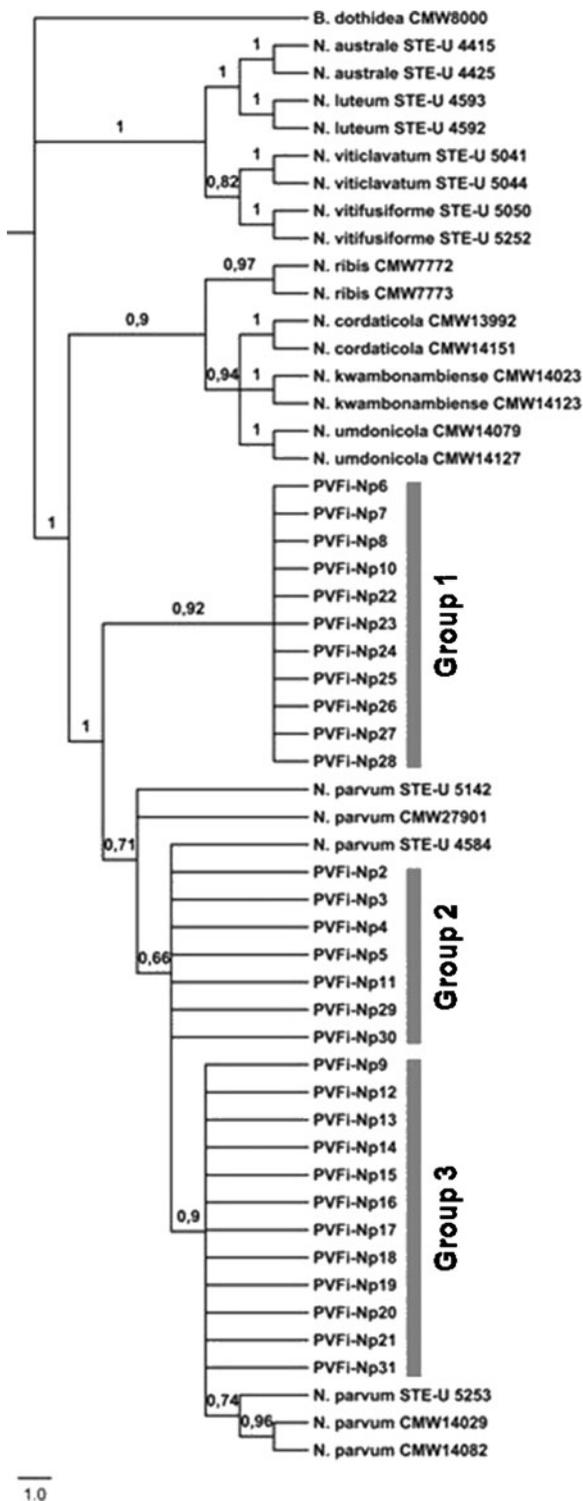
Fig. 2 Bayesian consensus phylogram (15002 sampled trees) reconstructed from the concatenated ITS-rDNA and EF1-α data sets of 30 *N. parvum* strains isolated in this study from standing vines and DGRC 2007. The MCMC analysis ran for 1×10^6 generations, with a sample frequency of 100 and a burn-in of 2500. Support values are posterior probabilities (the probability that the clade is correct, given the data and the model parameters). The tree was rooted to *B. dothidea* strain CMW8000 (acc. numbers: AY236949, AY236927). The bar represents 0.008 changes. The presence of three different phylogenetic groups within the isolated strains is highlighted with grey bars

BoitsA/BoitsB (Botryosphaeriaceae specific) and Pch1/Pch2 (*P. chlamydospora* specific), always discriminated correctly between target and non target DNAs (Tables 1 and 2). The theoretical sensitivity of the nested PCR assay with the primer set BoitsA/BoitsB when applied to the DNA in aqueous solution of *N. parvum* PVFi-Np9 was 1 pg, three dilution factors below the detection limit that was registered for this primer set when applied in single step PCR reaction (1 ng). When DNA of *Spencermartinsia viticola* CBS117009, the species of Botryosphaeriaceae that among those considered had shown the lowest theoretical identity with the primers BoitsA and BoitsB, was used, the detection limit was 10 pg and 10 ng in nested and single step PCR, respectively. In the case of the NprcA/NprcB primer set the detection limit was 10 and 100 fg of the DNA of *N. parvum* PVFi-Np9 in nested and single step PCR, respectively. In the case of the Pch1/Pch2 primer set it was 100 fg and 1 pg of the DNA of the strain PVFi-Pch36 in nested and single step PCR, respectively.

Evaluation of the PCR assays in vivo

With respect to fungal isolation, when the nested PCR assays were applied to the total DNA extracted from the naturally infected samples, a significant increase in the frequency of detection was always observed in the case of standing vines (Fig. 1a) and discoloured and non discoloured tissues of DGRC 2007 (Fig. 1b). The result of the survey carried out on pooled samples of woody tissues from 13 lots of DGRC 2008 revealed the presence of DNA of *N. parvum*/*N. ribis* complex, Botryosphaeriaceae and *P. chlamydospora* in 15, 29 and four out of 50 samples tested, respectively. As a whole ten, 12 and four lots out of 13 could be scored as infected by *N. parvum*/*N. ribis* complex, Botryosphaeriaceae and *P. chlamydospora*,

Fig. 3 Detection of *N. parvum*/*N. ribis* **a**, species in Botryosphaeriaceae **b** and *P. chlamydospora* **c** in naturally infected woody tissue samples (pooled samples) by means of three independent nested-PCR specific assays. From one to nine of pooled samples of woody tissues were taken from each of thirteen lots (1-13) of DGRC produced by five nurseries (A, B, C, D and E) in 2008 and from canes of *Vitis vinifera* subsp. *sylvestris* (Vvs). An asterisk above a pair of vertical bars indicates a statistically significant differences (P≤0.05) between lots, based on a "Fisher-Freeman-Halton" exact test analysis

respectively (Figs. 3a, b and c). Moreover, considering the results of each independent PCR assay, the only frequencies of detection that were found to be different were those relative to the *N. parvum*/*N. ribis* complex DNA (NprcA/NprcB assay) between different nurseries ($P=0.000$) and to the Botryosphaeriaceae DNA (BoitsA/BoitsB assay) between the different lots of DGRC 2008 produced by nursery A ($P=0.004$). Nevertheless, when the observed detection frequency of each of the 13 lots were compared in the same analysis (Fig. 3), they were not significant ($P=0.074$, $P=0.105$ and $P=0.424$ for the BoitsA/BoitsB, NprcA/NprcB and Pch1/Pch2 assays, respectively).

When the results obtained from each sample by each independent PCR assay were compared, evidence of the coexistence of DNA of different species within the same sample, was found (Fig. 4). Considering as a whole 43 (standing vines), 27 (DGRC 2007) and 32 (DGRC 2008) samples whose DNA could be amplified by at least one of the three assays, only the 18.6%, 25.9% and 43.8%, respectively were scored as positive to only one, meanwhile the 81.4% and the 74.1% and 56.2% tested positive to two or to all three independent assays. Both in the case of chip and pooled samples, all the DNA templates that tested positive to the NprcA/NprcB assay, also tested positive to the BoitsA/BoitsB assay or to both the BoitsA/BoitsB and Pch1/Pch2 assays (Fig. 4).

Chromatograms of good quality were obtained from 39 out of 42 BoitsA/BoitsB assay amplicons (three chromatograms could not be used given the presence of more than one sequence) and from all 15 NprcA/NprcB amplicons that were sequenced. The derived phylogenetic analysis showed that in only four cases (one sample from standing vines and three from pooled DGRC 2008) the BoitsA/BoitsB primer set had amplified the DNA of *N. parvum*/*N. ribis* complex (Fig. 5a and b). In the remaining 35 cases the DNA of *D. seriata* (eight out of nine samples from standing vines, five out of nine samples from
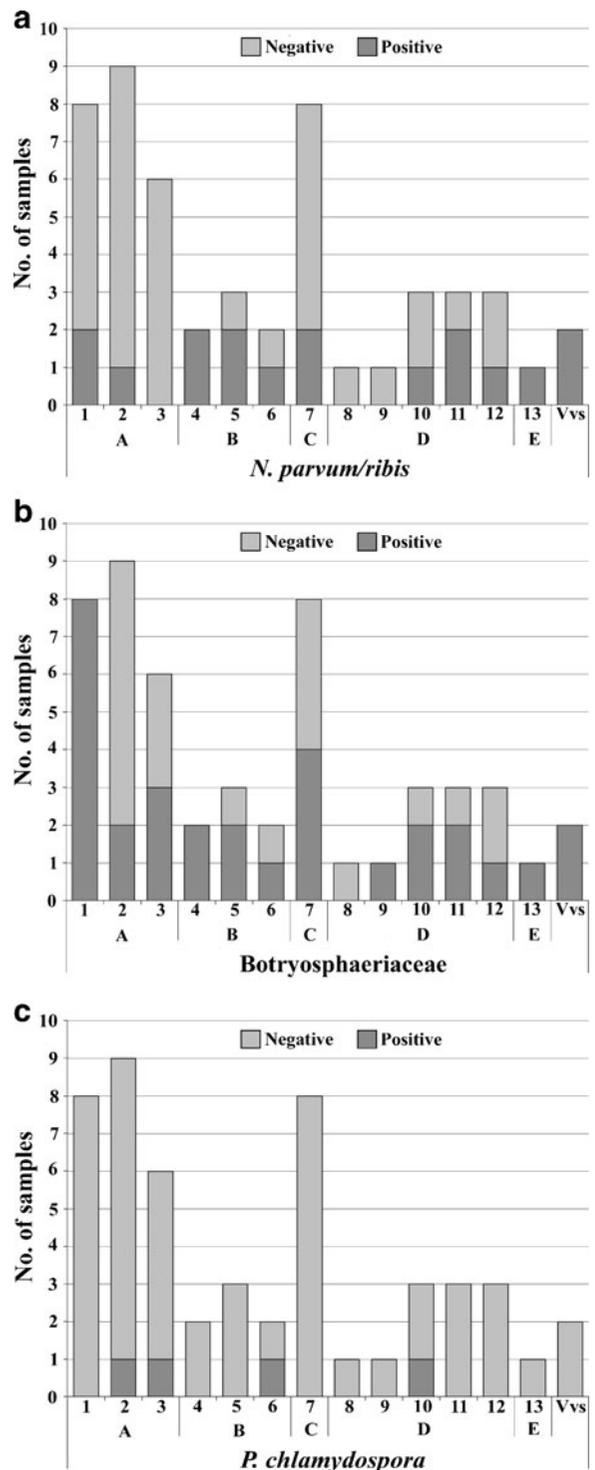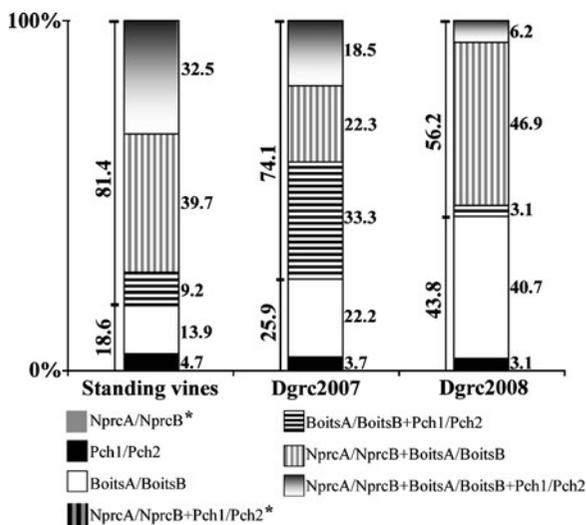
Fig. 4 Frequencies of detection of naturally infected woody tissue samples that tested positive to only one, two or to all three assays (NprcA/NprcB; BoitsA/B; Pch1/Pch2) used in this study. * No DNA template tested positive to only the NprcA/NprcB or to both the NprcA/NprcB and Pch1/Pch2 assays

DGRC 2007, nine out of 19 samples from DGRC 2008), *B. dothidea* (four samples out of nine from DGRC 2007, six out of 19 samples from DGRC 2008) and *Dothiorella sarmentorum* (one sample out of 19 from DGRC 2008) were present together with that of *N. parvum/N. ribis* complex in the sampled tissues (Fig. 5a and b). At last, the presence of *Diplodia mutila* and *Neofusicoccum australe* DNA together with that of *N. parvum/N. ribis* complex could be detected in the pooled samples of canes collected from *V. vinifera* subsp. *sylvestris* (Fig. 5a and b). According to sequencing and phylogenetic analysis, no false positive results were produced.

Discussion

In this study, two ITS-rDNA based nested PCR assays for the detection of Botryosphaeriaceae species associated with grapevine plants were developed. Thus, one (NprcA/NprcB assay) had a narrow target range to detect DNA of the *N. parvum/N. ribis* complex, whereas the other (BoitsA/BoitsB assay) had a target range sufficiently wide to amplify the DNA of all the Botryosphaeriaceae species that may colonize grapevine woody tissues. The two protocols were found to be efficient, sensitive and reliable when used with naturally infected grapevine tissues. The

Fig. 5 Bayesian consensus phylograms (15002 sampled trees) reconstructed from the portions of the ITS1-5.8-ITS2 nucleotide sequences amplified, from the total DNA extracted from 39 **a** and 15 **b** grapevine samples of different origin, by means of nested PCR using the BoitsA/BoitsB **a** or the NprcA/NprcB **b** assays, respectively. The MCMC analysis ran for 1×10^6 generations, with a sample frequency of 100 and a burn-in of 2500. Support values are posterior probabilities (the probability that the clade is correct, given the data and the model parameters). The tree was rooted to *Guignardia bidwelli* strain CBS111145 (EU683672). The bars represent 0.9 **a** and 1.0 **b** changes. Origin of DNA samples: DSV, discoloured woody tissues from standing vine; NDNSM, non discoloured woody tissues from nursery stock material; DNSM, discoloured woody tissues from nursery stock material; PNSM, pooled woody tissues from nursery stock material; PVvs, pooled woody tissues from *V. vinifera* subsp. *sylvestris* canes

NprcA/NprcB assay can cover the variability that exists within *N. parvum*. The BoitsA/BoitsB assay was able to detect the presence of at least six of the Botryosphaeriaceae species that have been associated with grapevine decline. Moreover, the molecular techniques developed here offer considerable advantages over culture-dependent detection in terms of time, reproducibility and low detection limits and thus may be useful for the early detection of low levels of infection of nursery stock material, or in epidemiological studies. With these tools, together with one specific for *P. chlamydospora* (Pch1/Pch2 assay, Edwards et al. 2007), the main agent of the Grapevine leaf stripe disease and Petri disease (Surico 2009), we have shown that in Italy nursery stock material may play a significant role as source of primary inoculum in the vineyards of not only *P. chlamydospora*, as reported previously (Retief et al. 2006; Edwards et al. 2007), but also of *N. parvum*, *D. seriata* and *B. dothidea*.

The results of our investigation allow some other interesting considerations that may be relevant for understanding the epidemiology of grapevine decline. With respect to direct isolation, the results obtained in this study by means of the molecular tools suggest that an intimate association between different agents of trunk diseases occur in nature both in standing vines and nursery propagating material. The concomitant presence of phytopathogenic fungi in the same wood symptoms of standing vines was observed in California by Úrbez-Torres et al. (2006) using isolations on agar media. In that study, 22% of the wood alterations were found to be infected by Botryosphaeriaceae species, but other fungi, such as *P. chlamydospora*, *Phaeoacremonium* spp., *Eutypa lata*, *Phomopsis viticola* and other
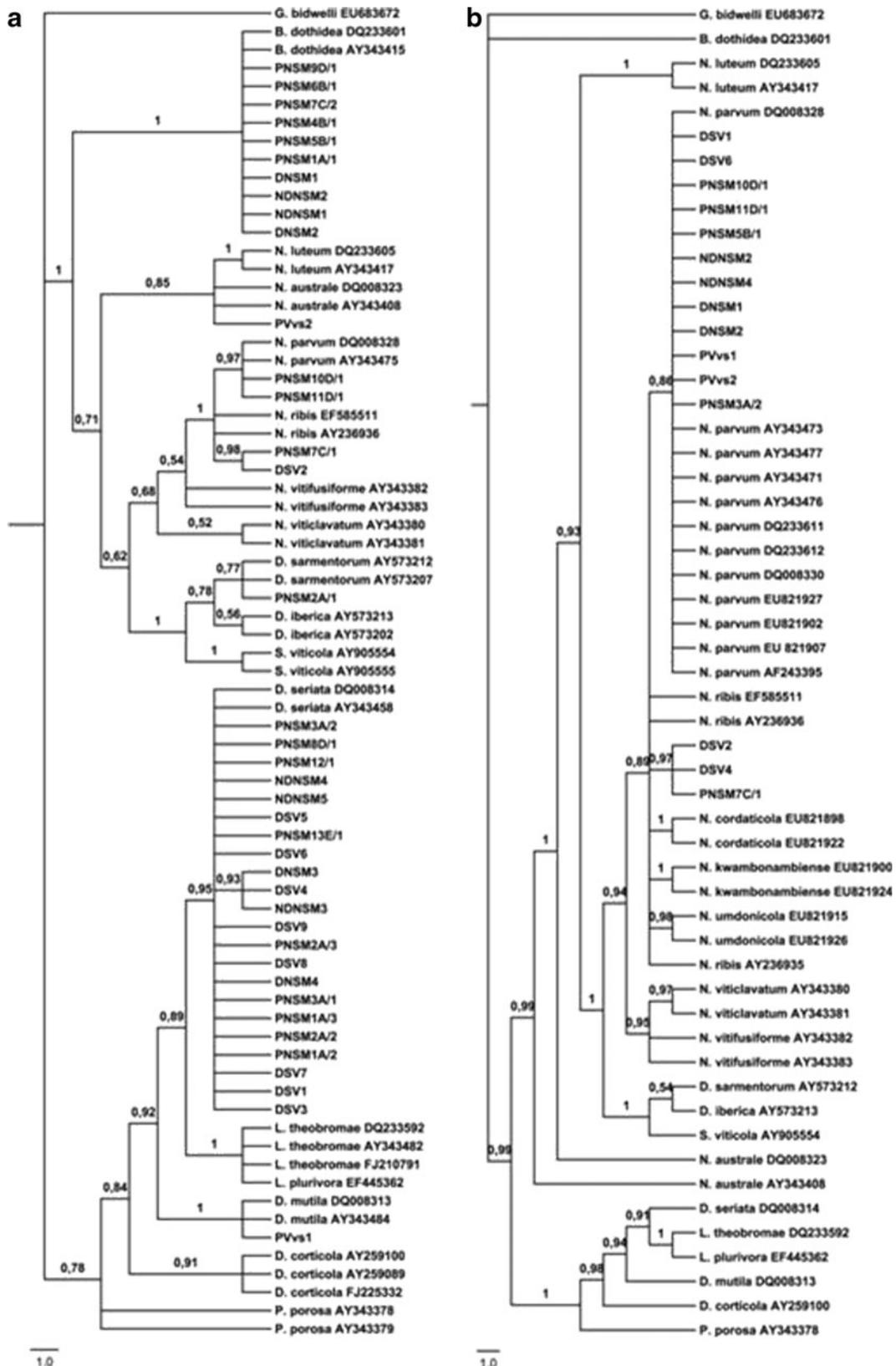
species of Ascomycetes were also detected. By means of direct fungal isolation, co-infections of nursery propagating material (grafted grapevines or rooted rootstocks) by several wood pathogens (*P. chlamydospora*, *Phaeoacremonium* spp., species in Botryosphaeriaceae, *Cylindrocarpon* spp. and *Phomopsis* spp.), were reported by Aroca et al. (2006) in Spain. Of the 97 plants that they found to be infected at the end of their survey, nearly 30% tested positive for the presence of two or more phytopathogenic species. In our study, in the case of standing vines, direct isolation indicated the concomitant presence of different species at the whole plant level, but when single wood discolourations were considered, the different species appeared to be spatially confined in different parts of the plants. Co-infection by Botryosphaeriaceae species and *P. chlamydospora* occurred in only three out of 54 of the analyzed samples whereas different Botryosphaeriaceae species were never isolated from the same wood discolouration. On the other hand, results of the analysis performed on the same material, using the three molecular protocols, indicated that DNA of two or all three targeted taxonomic groups were present in 81.4% of the samples that tested positive to at least one assay (43 out of 54). A similar situation was also observed in the case of nursery propagating material (DGRC 2007), where 27 of 45 samples tested positive to at least one assay and DNA of two or all three targeted taxonomic groups were present in 74.1% of the samples. Hence, our results support the increasing evidence that a broad range of taxonomically unrelated pathogens are independently contributing not only to a more or less non-specific decline of grapevines (Rolshausen et al. 2010), but perhaps also to more specific syndromes. This could be the case of the Grapevine leaf stripe disease (ex young esca) (Surico 2009), the aetiology of which has been conferred to more than one organism, including Botryosphaeriaceae species (Surico et al. 2006). In this regard, it has recently been shown (Martos et al. 2008; Evidente et al. 2010) that at least some of the Botryosphaeriaceae species isolated from grapevine wood produce the phytotoxic metabolites isosclerone (*N. parvum*) and exopolysaccharides (*N. parvum* and *N. luteum*). Interestingly, such compounds are produced also by *P. chlamydospora* and are currently considered to be involved in foliar symptom expression in Grapevine leaf stripe disease (Sparapano et al. 2000; Andolfi et al. 2009). Although the cause of the typical

foliar chloro-necrotic symptoms of this disease still needs to be fully elucidated (Surico et al. 2006), they could be the result, in toto or in part, of the synergistic action of the phytotoxic matabolites produced by species of Botryosphaeriaceae and *P. chlamydospora*.

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