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RESEARCH PAPERS

Characterization of *Neopestalotiopsis*, *Pestalotiopsis* and *Truncatella* species associated with grapevine trunk diseases in France

SAJEEWA S. N. MAHARACHCHIKUMBURA^{1,2}, PHILIPPE LARIGNON³, KEVIN D. HYDE^{4,5}, ABDULLAH M. AL-SADI² and ZUO-YI LIU^{1,*}

¹ Guizhou Key Laboratory of Agricultural Biotechnology, Guizhou Academy of Agricultural Sciences, Xiaohe District, Guiyang City, Guizhou Province, 550006 People's Republic of China

² Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, P.O. Box 34, Al-Khod 123, Oman

³ Institut Français de la Vigne et du Vin, Pôle Rhône-Méditerranée, 7 avenue Cazeaux, 30230 Rodilhan, France

⁴ Institute of Excellence in Fungal Research, Mae Fah Luang University, Tasud, Muang, Chiang Rai, 57100 Thailand

⁵ School of Science, Mae Fah Luang University, Tasud, Muang, Chiang Rai, 57100 Thailand

Summary. Pestalotioid fungi associated with grapevine wood diseases in France are regularly found in vine growing regions, and research was conducted to identify these fungi. Many of these taxa are morphologically indistinguishable, but sequence data can resolve the cryptic species in the group. Thirty pestalotioid fungi were isolated from infected grapevines from seven field sites and seven diseased grapevine varieties in France. Analysis of internal transcribed spacer (ITS), partial β -tubulin (TUB) and partial translation elongation factor 1-alpha (TEF) sequence data revealed several species of *Neopestalotiopsis*, *Pestalotiopsis* and *Truncatella* associated with the symptoms. Three *Neopestalotiopsis* spp. and one *Pestalotiopsis* sp. are reported for the first time associated with wood diseases in grapevine in France and worldwide, and include *Neopestalotiopsis asiatica*, *N. javaensis*, *Neopestalotiopsis* sp. and *Pestalotiopsis biciliata*. The sequence data indicate that *Truncatella angustata* was also associated with wood grapevine diseases in France; this species was previously reported on grapevine in Iran. The importance of controlling Pestalotioid fungi associated with wood grapevine diseases is discussed, which was previously considered of minor importance. These fungi are isolated from plants in nurseries before marketing.

Key words: cryptic species, pestalotioid fungi, trunk disease, *Vitis vinifera*.

Introduction

Vitis vinifera L. (grapevine) is native to the Mediterranean region, central Europe, and southwestern Asia, and is widely grown for its fruits throughout the world (Terral *et al.*, 2010). The ancient Greeks introduced grape cultivation to Europe in the Minoan age (Phillips, 2000a). France is among the top five countries in the world in grape production, with a total production of 5.5 million tonnes in 2013 (FAO, 2016).

Grapevine trunk diseases impact the economic production and longevity of vineyards, and are con-

sidered to be the most destructive diseases of grapevines (Hofstetter *et al.*, 2012; Bruez *et al.*, 2013; Gramaje *et al.*, 2016; Larignon 2016). These diseases have severe effects on the perennial plant parts, mainly the trunks of vines. In France, three main diseases, esca disease, Botryosphaeria dieback and eutypa dieback, are the most destructive, and have long been known wherever grapes are grown (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Bertsch *et al.* 2013). A complex of fungal species, especially *Fomitiporia mediterranea* Fischer, *Phaeomoniella chlamydospora* (W. Gams *et al.*) Crous & W. Gams and *Phaeoacremonium minimum* (Durieu & Mont.) D. Gramaje *et al.*, are associated with esca disease. Botryosphaeriaceae spp. cause Botryosphaeria dieback, *Eutypa lata* (Pers.) Tul.

Corresponding author: Z.-Y. Liu
E-mail: gzliuzuoyi@163.com

& C. Tul. causes Eutypa dieback, and *Diaporthe ampelina* (Berk. & M.A. Curtis) R.R. Gomes *et al.* causes Phomopsis cane and leaf spot. (Phillips, 2000b; Gramaje *et al.*, 2016; Larignon 2016). All these diseases can be observed in the same plant and presented a fungal and bacterial microflora accompanying these pathogen agents (Bruez *et al.*, 2014, 2015). Symptoms of these diseases include stunted shoots with shortened internodes, defoliated shoots, dieback of one or more shoots accompanied by leaf drop, leaf stripe and apoplectic forms and woody necrosis (brown or grey wedge-shaped necrosis, white rot, brown stripe, black dots) (Bertsch *et al.*, 2013). These trunk diseases trigger gradual decline of grapevines due to inner alterations of the wood, and result in decreasing yield and quality of grapes (Moreno-Sanz *et al.*, 2013). Many pathogens infect mature grapevines. However, there are also many records of pathogens infecting young grapevines (Bertelli *et al.*, 1998; Giménez-Jaime *et al.*, 2006; Halleen *et al.*, 2006; Dubrovsky and Fabritius, 2007; Schroers *et al.*, 2008; Larignon *et al.*, 2015).

Neopestalotiopsis, *Pestalotiopsis* and *Truncatella* belong to the order Xylariales and are generally known as pestalotioid fungi (Maharachchikumbura *et al.*, 2014). These taxa are significant phytopathogens causing postharvest fruit rot and trunk diseases in grapevines in many countries (Arzanlou *et al.*, 2013; Jayawardene *et al.*, 2015), and were the second most common taxa isolated from grapevine cankers in Texas, following *Botryosphaeria* spp. (Úrbez-Torres *et al.*, 2011). However, recognition of species, using phenotypes, is difficult, as morphological characters used to differentiate species are limited, plastic and vary between hosts and environments. In the present study, we surveyed diseases in vine growing regions in France constantly observing wood disease, and isolated the associated fungi. Based on an initial microscopic investigation of the conidia of isolates, the organisms were identified as pestalotioid fungi. We identified the pestalotioid fungi to species level based on ITS, TUB and TEF sequence data. Knowledge about pestalotioid fungi associated with grapevines will help provide a basis for developing management strategies of these pathogens.

Materials and methods

Sample collection, isolation and identification

Diseased grapevine samples characterized by leaf stripes and defoliated shoots were obtained

from various viticultural districts of France (Aquitaine, Burgundy, Champagne, Languedoc-Roussillon, Rhône-Alpes) between January 2010 to January 2012. Plant material was also obtained from nurseries in Midi-Pyrénées and PACA. Isolations were made from tissues according to Larignon and Dubos, (1997). Isolation plates were incubated at room temperature for 3 weeks. Colonies grown on malt agar (MA) plates were transferred to MA and stored at 4°C for further study.

These fungal colonies were then grown on potato dextrose agar (PDA) incubated at 25°C for 7 to 10 d and were initially identified using morphological traits following Maharachchikumbura *et al.* (2012; 2014).

Molecular phylogenies

DNA extraction, PCR amplification, and DNA sequencing

Total genomic DNA was extracted from fresh fungal mycelia (500 mg), scraped from the margin of a colony on a PDA plate incubated at 25°C for 7–10 d (Guo *et al.*, 2000). The ITS, TUB and TEF regions were amplified using primer pairs ITS4/ITS5 (White *et al.*, 1990), BT2A/BT2B (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), and EF1-526F or EF728F/EF1-1567R or EF2 (Carbone and Kohn, 1999; O'Donnell *et al.*, 1998; Rehner, 2001). Polymerase chain reaction (PCR) was performed for each sample with the 25 µL reaction system consisting of 19.5 µL of double-distilled water, 2.5 µL of 10× Taq buffer with MgCl₂, 0.5 µL of dNTP (10 mM each), 0.5 µL of each primer (10 µM), 0.25 µL of Taq DNA polymerase (5 U µL⁻¹), and 1.0 µL of DNA template. The thermal cycling programme followed that of Maharachchikumbura *et al.* (2012).

Three different datasets were used to estimate three phylogenies: a *Neopestalotiopsis* tree, *Pestalotiopsis* tree (ITS, TUB and TEF), and a *Truncatella* tree. The *Neopestalotiopsis* and *Pestalotiopsis* trees were based on combined datasets, and the *Truncatella* tree was based on an ITS dataset. Sequences generated in this study (Table 1) were supplemented with additional sequences obtained from GenBank, based on blast searches and the literature. Multiple sequence alignments were generated with MAFFT v. 7 (<http://mafft.cbrc.jp/alignment/server/index.html>); the alignments were visually improved with Mesquite v. 2.75 (Maddison and Maddison, 2011) and MEGA v. 5.2.2 (Kumar *et al.*, 2012) or BioEdit

Table 1. Collection details and GenBank accession numbers of isolates includes in this study.

Species	Culture accession No. (MFLUCC)	Variety	Location ¹	Symptom ²	Collector ³	ITS	TUB	TEF
<i>Neopestalotiopsis asiatica</i>	12-0573	Mourvèdre	P (nursery)	*	L	KX816909	KX816937	KX816878
<i>N. asiatica</i>	12-0575	Gamay	R (vineyard)	Leaf stripes	L	KX816901	KX816930	KX816871
<i>N. asiatica</i>	12-0585	Cabernet-Sauvignon	A (vineyard)	Leaf stripes	L	KX816923	KX816951	KX816893
<i>N. asiatica</i>	12-0586	Cabernet-Sauvignon	A (vineyard)	Leaf stripes	L	KX816910	KX816938	KX816879
<i>N. asiatica</i>	12-0591	Rootstock 110R	MP (nursery)	*	L	KX816917	KX816945	KX816886
<i>N. asiatica</i>	12-0597	Rootstock 110R	MP (nursery)	*	L	KX816898	KX816928	KX816869
<i>N. asiatica</i>	12-0600	Rootstock 110R	MP (nursery)	*	L	KX816911	KX816939	KX816880
<i>N. asiatica</i>	12-0617	Sauvignon	MP (nursery)	*	L	KX816915	KX816943	KX816884
<i>N. asiatica</i>	12-0623	Rootstock 110R	MP (nursery)	*	L	KX816908	KX816936	KX816877
<i>N. asiatica</i>	12-0624	Rootstock 110R	MP (nursery)	*	L	KX816921	KX816949	KX816891
<i>N. asiatica</i>	12-0626	Rootstock 110R	MP (nursery)	*	L	KX816922	KX816950	KX816892
<i>N. asiatica</i>	12-0627	Rootstock 110R	MP (nursery)	*	L	KX816924	KX816952	KX816894
<i>N. asiatica</i>	12-0628	Rootstock 110R	MP (nursery)	*	L	KX816907	KX816935	KX816876
<i>N. jacaensis</i>	12-0594	Rootstock 110R	MP (nursery)	*	L	KX816905	KX816933	KX816874
<i>Neopestalotiopsis</i> sp.	12-0601	Rootstock 110R	MP (nursery)	*	L	KX816902	KX816931	KX816872
<i>Neopestalotiopsis</i> sp.	12-0603	Rootstock 110R	MP (nursery)	*	L	KX816899	KX816929	KX816870
<i>Neopestalotiopsis</i> sp.	12-0604	Rootstock 110R	MP (nursery)	*	L	KX816918	KX816946	KX816888
<i>Neopestalotiopsis</i> sp.	12-0608	Rootstock 110R	MP (nursery)	*	L	KX816912	KX816940	KX816881
<i>Neopestalotiopsis</i> sp.	12-0614	Rootstock 110R	MP (nursery)	*	L	KX816919	KX816947	KX816889
<i>Neopestalotiopsis</i> sp.	12-0622	Sauvignon	MP (nursery)	*	L	KX816913	KX816941	KX816882
<i>Pestalotiopsis biciliata</i>	12-0577	Pinot noir	B (vineyard)	Leaf stripes	L	KX816903	-	-
<i>P. biciliata</i>	12-0578	Pinot noir	B (vineyard)	Defoliated shoots	L	KX816916	KX816944	KX816885
<i>P. biciliata</i>	12-0579	Pinot noir	B (vineyard)	Defoliated shoots	L	KX816914	KX816942	KX816883
<i>P. biciliata</i>	12-0592	Rootstock 110R	MP (nursery)	*	L	KX816896	KX816927	KX816887
<i>P. biciliata</i>	12-0598	Rootstock 110R	MP (nursery)	*	L	KX816920	KX816948	KX816890
<i>P. biciliata</i>	12-0607	Rootstock 110R	MP (nursery)	*	L	KX816895	KX816926	-
<i>P. biciliata</i>	12-0609	Rootstock 110R	MP (nursery)	*	L	KX816904	KX816932	KX816873
<i>P. biciliata</i>	12-0620	Sauvignon	MP (nursery)	*	L	KX816906	KX816934	KX816875
<i>Truncatella angustata</i>	12-0580	Pinot noir	C (vineyard)	Defoliated shoots	S	KX816925	-	-
<i>T. angustata</i>	12-0587	Mourvèdre	LR (vineyard)	Defoliated shoots	L	KX816897	-	-

¹ P: PACA, R: Rhône-Alpes, A: Aquitaine (Gironde), MP: Midi-Pyrénées, B: Burgundy (Côte d'Or), C: Champagne (Marne), LR: Languedoc-Roussillon (Gard).

² *: Grafted plants in nursery with no symptoms.

³ L: Laignon, S: Spagnolo.

ITS: internal transcribed spacers and intervening 5.8S nrDNA; TUB: partial beta-tubulin gene; TEF: partial translation elongation factor 1-alpha gene.

v. 7.0.5.2 (Hall, 1999). Phylogenetic analyses of the sequence data consisted of Bayesian Inference (BI) and Maximum Likelihood (ML) analyses of both the individual data partitions and the combined aligned dataset. Ambiguously aligned regions were excluded from all analyses and gaps were treated as “missing data” in the parsimony analyses. Suitable models for the Bayesian analyses were first selected using models of nucleotide substitution for each gene, as determined using MrModeltest v. 2.2 (Nylander, 2004), and included for each gene partition. The Bayesian analyses (MrBayes v. 3.2.1; Ronquist *et al.*, 2012) of four simultaneous Markov Chain Monte Carlo (MCMC) chains were run from random trees for 10,000,000 generations and sampled every 1,000 generations. The temperature value was lowered to 0.15, burn-in was set to 0.25, and the run was automatically stopped as soon as the average standard deviation of split frequencies reached less than 0.01. A ML analysis was performed using raxmlGUI v. 1.3 (Silvestro and Michalak, 2011). The optimal ML tree search was conducted with 1,000 separate runs, using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR+GAMMA substitution model. The resulting trees were printed with FigTree v. 1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>) and the layout was completed with Adobe Illustrator CS v. 6.

Results

A total of eight pestalotioid isolates were obtained from symptomatic plants (leaf stripe, defoliated shoots) of different grapes varieties, and 22 were obtained from grafted plants in grapevine nurseries in France. Other fungi were isolated from the symptomatic plants are known as associated with esca disease (*Fomitiporia mediterranea*, *Phaeomoniella chlamydospora*), Botryosphaeria dieback (*Diplodia seriata*) or Phomopsis cane and leaf spot (*Diaporthe ampelina*). Results of isolated pestalotioid fungi from grapes are shown in Table 1. Based on the initial morphological characterization, these isolates belonged to three genera; *Neopestalotiopsis* (20 isolates), *Pestalotiopsis* (eight isolates) and *Truncatella* (two isolates). Member of the genus *Pestalotiopsis* can be easily identified based on five-celled, fusiform conidia, with three light concolourous median cells, with hyaline end

cells. *Neopestalotiopsis* can easily be distinguished from *Pestalotiopsis* by its five-celled, fusiform conidia, with versicolorous median cells. *Truncatella* was easily identified based on its four-celled, fusiform conidia. The combined gene tree of isolates obtained in this study consists of the strains that have ex-types or ex-epitypes, plus the isolates obtained during the study.

Species relationships in *Neopestalotiopsis* are shown in Figure 1. The combined ITS, TUB, and TEF dataset comprises strains representing 76 taxa of *Neopestalotiopsis* with *Pestalotiopsis trachicarpicola* (IFRDCC 2440) as the outgroup taxon, and the manually adjusted dataset comprised 1,421 characters including gaps. The GTR+I model with a proportion of invariable sites for ITS and the HKY+G model with gamma-distributed rate model for TUB and the GTR+I+G model with inverse gamma rate were selected for TEF and included for each gene partition in MrBayes. For the combined genes, ML and BI consensus trees revealed the same phylogenetic relationships between the significantly supported clades. The *Neopestalotiopsis* isolates obtained in this study clustered with two previously published species, namely, *N. javaensis* (one isolate) and *N. asiatica* (13 isolates). The *N. javaensis* (isolate MFLUCC 12-0594) was obtained from the Midi-Pyrénées and the *N. asiatica* isolates were from the Aquitaine (Gironde), Midi-Pyrénées, Provence-Alpes-Côte d’Azur and Rhône-Alpes regions of France. Six isolates from the present study obtained from the Midi-Pyrénées, and two isolates derived from Jayawardeneet *al.* (2016), formed a weakly support clade with *N. vitis*. Since there is slight variation in sequence data, we prefer to maintain them as *Neopestalotiopsis* sp. until additional collections and cultures become available.

To clarify the *Pestalotiopsis* species associated with grapevine trunk disease, a combined alignment of ITS, TUB and TEF sequence data (including the outgroup *Neopestalotiopsis saprophytica*; MFLUCC 12-0282) for 63 species generated 1,520 characters including alignment gaps (Figure 2). Dirichlet base frequencies and the GTR+I+G model with inverse gamma-distributed rate for ITS and HKY+I+G models with inverse gamma-distributed rates were selected for TUB and TEF and set in MrBayes. The eight *Pestalotiopsis* isolates obtained in this study clustered with type strains of *P. biciliata* (CBS 124463). These isolates were derived from the Aquitaine (Gironde), Burgundy (Côte d’Or), Midi-Pyrénées, Provence-

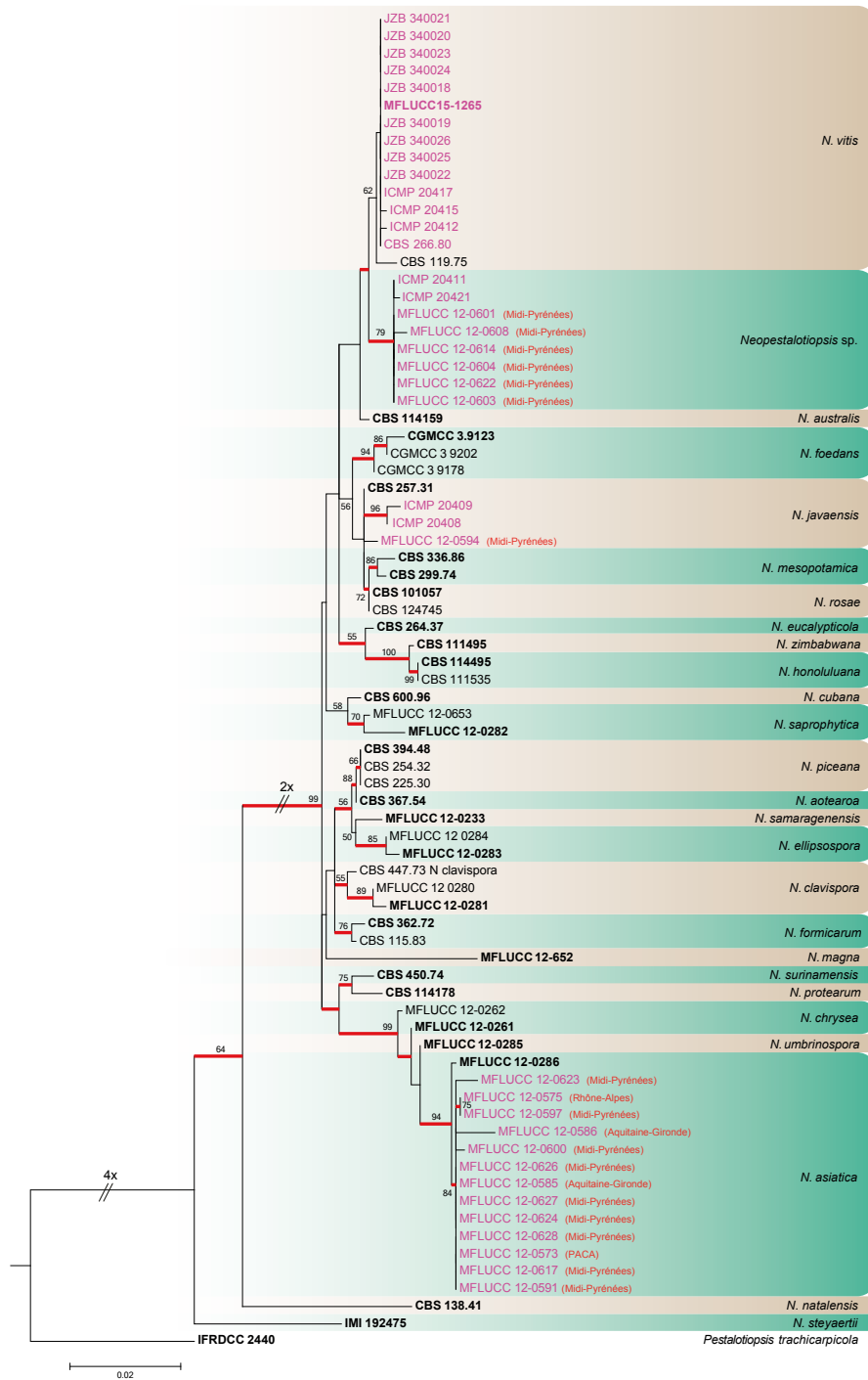


Figure 1. Consensus phylogram (50% majority rule) of 1,000 trees resulting from a RAxML analysis of the (ITS+TUB+TEF) alignment of the analysed *Neopestalotiopsis* sequences. Red-thickened lines indicate Bayesian posterior probabilities (PP) above 90% and RAxML bootstrap support values (MLB) above 50% are given at the nodes. Sequences derived from ex-type strains are printed in bold type, and isolates derived from grapevine are printed in magenta and followed by the place of origin in France. The species name is indicated to the right of each clade. The scale bar represents the expected number of changes per site. The tree was rooted to *Pestalotiopsis trachicarpicola* (IFRDCC 2440).

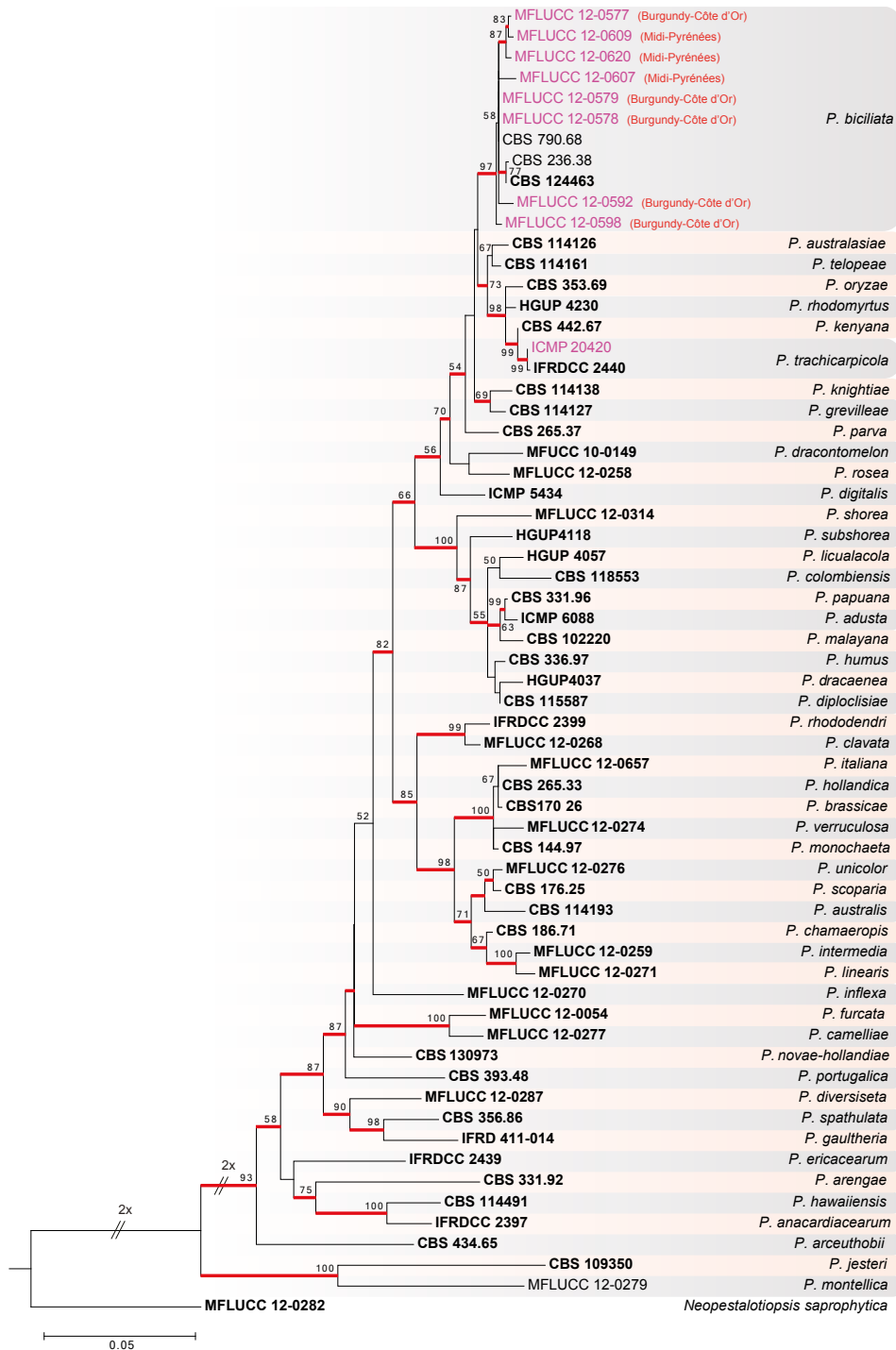


Figure 2. Consensus phylogram (50% majority rule) of 1,000 trees resulting from a RAxML analysis of ITS+TUB+TEF sequence data from *Pestalotiopsis* species. Red-thickened lines indicate Bayesian posterior probabilities (PP) above 50% and RAxML bootstrap support values above 90% are given at the nodes. Sequences derived from ex-type are printed in bold type, and isolates derived from grapevine are printed in magenta and followed by the place of origin in France. The species name is indicated to the right of each clade. The scale bar represents the expected number of changes per site. The tree is rooted to *Neopestalotiopsis saprophytica* (MFLUCC 12-0282).

Alpes-Côte d'Azur and Rhône-Alpes regions of France.

The ITS alignment was used to identify the *Truncatella* species associated with trunk diseases in grapevine. The alignment comprised 37 strains (including the outgroup taxon *Pestalotiopsis malayana*) and the manually adjusted dataset comprised 547 characters including gaps. Dirichlet base frequencies and the K80+G model were recommended by the MrModeltest analysis and used in the Bayesian analysis. Two *Truncatella* isolates were isolated from symptomatic vineyards in Champagne (Marne) and Languedoc-Roussillon (Gard) regions. Based on the phylogenetic tree, these two isolates are identified as *Truncatella angustata*. However the genus *Truncatella* is probably polyphyletic and species belong to the genus clusters in two different clades in the family *Bartalaniaceae*.

Discussion

Although most pestalotioid fungi are phenotypically challenging to identify, initial identification based on morphology allow them to be classified into genera and some into species (Hyde *et al.*, 2014). Conidium morphology is an extensively used taxonomic character in these groups of fungi (Steyaert, 1949; Guba, 1961; Nag Raj, 1993). However, morphological characters are plastid and vary based on host and environment. Phenotypic characteristics overlap which makes it difficult to segregate morphologically equivocal taxa. However, sibling taxa can be better resolved using sequence data. In this study, we used combined analyses of ITS, TUB and TEF datasets to characterize *Neopestalotiopsis* and *Pestalotiopsis* species associated with grapevines in France. Phylogenetic analyses of DNA sequence data from grapes found no relationships between host variety or geographic sources. Phylogenetic species recognition was the most effective method for differentiation of pestalotioid fungi because they were closely related in terms of morphology and biology. The ecology of pestalotioid fungi is little studied, there being a lack of data on geographical distribution and host specificity. We recommended further research on these aspects of this group of pathogens.

The genus *Truncatella* was introduced by Steyaert (1949) to accommodate species having three-septate conidia, which previously belonged to *Pestalotia*. This genus was typified by *T. truncata* (Lév.) Steyaert.

Pestalotia is typically associated with plants as endophytes or pathogens (Shoemaker *et al.*, 1989). Our phylogenetic analyses (Figure 3) suggest that there might be two distinct clades in *Truncatella* and a new genus may need to be introduced for the *Truncatella sensu lato*. The same was observed by Jeewon *et al.*, (2002) and Li *et al.*, (2015); *Truncatella* is paraphyletic with *Bartalinia* sharing a common ancestor and has been placed in *Bartalaniaceae* (Senanayake *et al.*, 2015). The ex-type culture of this genus (*T. truncata*) is unavailable, and therefore, it is difficult to recognize the type lineage of *Truncatella*. Thus, recollecting material from type localities and isolating the organism into pure culture are essential, to provide further taxonomic and phylogenetic information on *Truncatella*.

Little information is available concerning the pathogens responsible for grapevine wood diseases in grapevines (Gramaje and Armengol, 2011). For years, *Eutypa lata*, *Fomitiporia mediterranea*, *Botryosphaeria* spp. and several other fungi belonging to Dothideomycetes and Sordariomycetes have been considered as the main fungal species involved in wood disease in grapevine (Larignon and Dubos, 1997; Phillips 2002). However, pestalotioid fungi have recently been isolated from grapevines, and have been shown to be pathogenic (Úrbez-Torres *et al.*, 2009; Úrbez-Torres and Gubler, 2009; Trouillas *et al.*, 2010; Arzanlou *et al.*, 2013; Jayawardene *et al.*, 2016). Grapevine trunk diseases reduce yield and quality of grapes, even leading to partial or total death of individual plants. Therefore, initial identification of the causal agent is essential for early control strategies to be implemented. The isolation of several species from plants in nurseries suggests that their dispersal is likely within or on host vegetative propagation material, as with other fungi associated with grapevine wood diseases (Gramaje and Armengol, 2011).

Pestalotioid fungi have been reported as pathogens on a variety of grapevine cultivars, causing diseases including grapevine dieback, fruit rot, postharvest disease and severe defoliation (Urbez-Torres *et al.*, 2009; Jayawardene *et al.*, 2015). These fungi affect all plant parts including leaves, canes, wood, berries and flowers (Maharachchikumbura *et al.*, 2011; Jayawardene *et al.*, 2015). *Pestalotiopsis menezesiana* (Bres. & Torr.) Bissett. and *Pestalotiopsis uvicola* (Spegazzini) Bissett are the most common species recorded from grapevine around the world (Guba, 1961; Sergeeva *et al.*, 2005; Urbez-Torres *et al.*, 2009, 2012). Most of

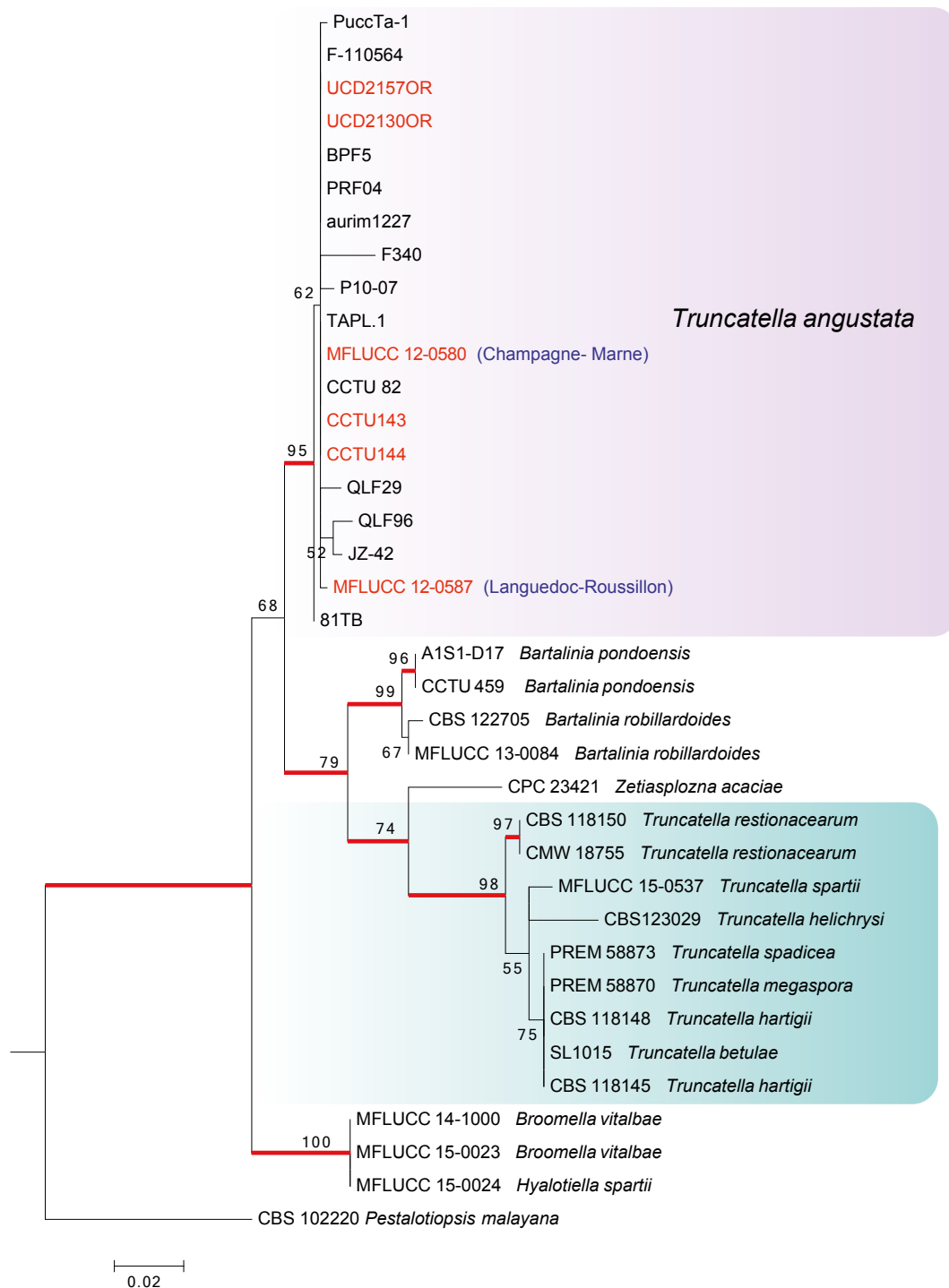


Figure 3. Consensus phylogram (50% majority rule) of 1,000 trees resulting from a RAXML analysis of the ITS sequence data of *Truncatella* and other genera in the family *Bartalaniaceae*. Red-thickened lines indicate Bayesian posterior probabilities (PP) above 90%, and RAXML bootstrap support values (MLB) above 50% are given at the nodes. The scale bar represents the expected number of changes per site. Strain accession numbers are followed by the species name (isolates derived from grapevine are printed in red). The tree is rooted to *Pestalotiopsis malayana* (CBS 102220).

these identifications were based on morphological characters. However, these two species lack ex-type strains, and therefore have not been included in the phylogenetic analysis in the present study.

Pestalotiopsis wicola has similar morphology to *P. trachicarpicola*, but the conidiogenous cells of *P. wicola* are ampuliform and the apical appendages often form closely aggregated crests, which do not occur in *P. trachicarpicola* (Jayawardane et al., 2015). *Pestalotiopsis menezesiana* is characterized by conidiogenous cells each with none to two closely spaced annular scars (Bissett, 1982). These were not observed in the species recorded in the present study. *Pestalotiopsis menezesiana* has versicolorous median cells, and thus this species probably belongs to the *Neopestalotiopsis*.

Maharachchikumbura et al., (2011) showed that many of the *Pestalotiopsis* species names in GenBank clustered throughout the phylogram and none of them are linked to any type material. Therefore, it is not possible to use gene sequences in GenBank to reliably clarify species names unless they are derived from ex-types. To our knowledge, this is the first report showing that *Neopestalotiopsis asiatica*, *Neopestalotiopsis javaensis* and *Pestalotiopsis biciliata* are associated with trunk grapevine disease.

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