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 considered 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.
& C. Tul. causes Eutypa dieback, and Diaporthe am-pelina (Berk. & M.A. Curtis) R.R. Gomes et al. caus-es Phomopsis cane and leaf spot. (Phillips, 2000b; Gramajé 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 (Bruzé 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, 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 included in this study.

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<th>Species</th>
<th>Culture accession No. (MFLUCC)</th>
<th>Variety</th>
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<th>Symptom2</th>
<th>Collector3</th>
<th>ITS</th>
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<td>T. angustata</td>
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<td>Mourvèdre</td>
<td>LR (vineyard)</td>
<td>Defoliated shoots</td>
<td>L</td>
<td>KX816897</td>
<td>-</td>
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</table>

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: Larignon, 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 easily be distinguished from Pestalotiopsis by its five-celled, fusiform conidia, with versusicolorous median cells. Truncatella was easily identified based on its four-celled, fusiform conidia. The combined gene tree of 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 trachicarpica (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. javensis (one isolate) and N. asiatica (13 isolates). The N. javensis (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+G model 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 trachicarpica (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 Bartalanaceae.

**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 Bartalanaceae (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 (Úrbez-Torres et al., 2009; Jayawardene et al., 2015). These fungi affect all plant parts including leaves, canes, wood, berries and flowers (Maharachchikumbra 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; Úrbez-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 uvicola* has similar morphology to *P. trachicarpicola*, but the conidiogenous cells of *P. uvicola* 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 javaeensis* and *Pestalotiopsis biciliata* are associated with trunk grapevine disease.

**Acknowledgements**

The authors thank the Featured Microbial Resources and Diversity Investigation in Southwest Karst area (2014FY120100) and the Mushroom Research Foundation, Chiang Mai, Thailand, for funding this research. Philippe Larignon thanks the FranceAgriMer and Casdar for financial support.

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*Accepted for publication: September 26, 2016*

*Published online: January 9, 2017*