

STUDY OF THE PORTUGUESE POPULATIONS OF POWDERY MILDEW FUNGUS FROM DIVERSE GRAPEVINE CULTIVARS (*VITIS VINIFERA*)

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Abstract

Aims: The main goal of this work was to describe the genetic diversity and population structure of Portuguese isolates from *Erysiphe necator* using a new approach for the sampling of DNA from this pathogen.

Methods and results: The present study was conducted in the main demarcated wine regions of Portugal, where leaf and berry samples were collected for further genetic analysis. After DNA extraction, the Internal Transcribed Spacer from the 18S ribosomal RNA (ITS), the Intergenic Spacer (IGS), and the β -tubulin gene were successfully amplified and sequenced. The populations of *E. necator* are structured into two genetically distinct groups (A and B), with the majority of the analyzed samples belonging to group B.

Conclusion: Regardless of its geographical origin and the *Vitis vinifera* cultivar, the population of *E. necator* presents low genetic diversity.

Significance and impact of the study: The present work will certainly help to increase the vineyard productivity through a better definition of preventive measures and a potential decrease in the direct and indirect costs associated with the use of fungicides.

Key words: disease control, *Erysiphe necator*, genetic diversity, powdery mildew, *Vitis vinifera*

Résumé

Objectifs: L'objectif principal de ce travail était de décrire la diversité et la structure de la population génétique des isolats d'*Erysiphe necator* portugais en utilisant une nouvelle approche pour la collecte d'échantillons d'ADN à partir de ce pathogène.

Méthodes et résultats: L'étude a été menée dans les principales régions viticoles portugaises, où des échantillons de feuilles et de baies ont été prélevés pour effectuer les analyses génétiques. Après extraction de l'ADN, les régions intergéniques de l'ARN ribosomique 18S (ITS), l'espace intergénique et le gène de la β -tubuline ont été amplifiés et séquencés avec succès. Les populations d'*E. necator* sont structurées en deux groupes génétiquement distincts (A et B). La majorité des échantillons analysés correspondaient au groupe B.

Conclusion: Indépendamment de leur provenance géographique et du cultivar de *Vitis vinifera*, la population d'*E. necator* présente une faible diversité génétique.

Signification et impact de l'étude: Le présent travail contribuera certainement à augmenter la productivité des produits des vignobles grâce à une meilleure définition des mesures préventives et à une diminution potentielle des coûts directs et indirects liés à l'utilisation de fongicides.

Mots clés: lutte contre les maladies, *Erysiphe necator*, diversité génétique, oïdium, *Vitis vinifera*

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INTRODUCTION

The *Vitis* genus includes around 60 species of perennial plants belonging to the Vitaceae family, predominantly from the Northern Hemisphere. *Vitis vinifera*, native to the Mediterranean region and Central Asia, is the most cultivated *Vitis* species in Europe. According to the Food and Agriculture Organization (FAO, 2014), grapevine is the leading fruit crop, with more than seven million ha of vineyards grown worldwide. Although grapevines grow within a broad range of climates, most vineyards are planted in temperate areas, most of them occurring in Europe. Portugal, with a vineyard area of nearly 243,000 ha (OIV, 2014), is ranked fifth in European wine producing countries and 11 worldwide (FAO, 2014). In Portugal, there are eight demarcated regions and 33 protected designations of origin (Denominação de Origem Controlada - DOC), which represent in 2012 more than 250 grape varieties (OIV, 2014). These regions are also very different in terms of weather, soils, vine-growing systems, crop growing techniques, disease incidence and, mostly, yield and wine quality.

Grapevines are highly susceptible to various pathogens that cause economically devastating diseases and require intensive use of fungicides for their control (Glawe, 2008). Among these pathologies, powdery mildew (*Erysiphe necator*), downy mildew (*Plasmopara viticola*), and *Botrytis* bunch rot (*Botrytis cinerea*) are the most important. The highly susceptible nature of *V. vinifera* to powdery mildew shows that this species lacks resistance mechanisms to defend itself against this pathogen. Symptoms of this disease can be detected during the vegetative growth on foliage, flowers, fruit, and canes. Severe infection can debilitate grapevines, reduce net photosynthesis, retard ripening, degrade grape and wine quality, and cause dramatic production losses (Halleen and Holz, 2001; Gadoury *et al.*, 2007).

Powdery mildew, caused by the biotrophic ascomycete *E. necator* (Schwein) Burrill, is the most economically important grapevine foliage disease throughout the world (Deliere *et al.*, 2010; Dufour *et al.*, 2011). Due to its obligatory and biotrophic nature, the study of the genetic diversity and population structure of this fungal pathogen has been difficult (Stummer *et al.*, 2000). Using different molecular markers, many attempts have been made worldwide to perform such studies, namely in North America (Brewer and Milgroom, 2010; Frenkel *et al.*, 2010; Brewer *et al.*, 2011; Frenkel *et al.*, 2012), Australia (Evans *et al.*, 1996; Evans *et al.*, 1997;

Stummer *et al.*, 2000; Halleen and Holz, 2001; Stummer and Scott, 2003) and Europe (Delye *et al.*, 1997; Delye *et al.*, 1999; Miazzi *et al.*, 2003; Cortesi *et al.*, 2004; Cortesi *et al.*, 2005; Péros *et al.*, 2005; Amrani and Corio-Costet, 2006; Núñez *et al.*, 2006; Cortesi *et al.*, 2008; Miazzi *et al.*, 2008; Montarry *et al.*, 2008; Montarry *et al.*, 2009). These works reported that populations of *E. necator*, both in Europe and Australia, are structured into two genetically distinct groups (A and B) that were finally identified in many grapevine-growing countries (Delye *et al.*, 1997; Evans *et al.*, 1997). Group A, only observed in a reduced number of cultivars (Carignan, Cinsault, and Villard Noir), is genetically less diverse and has been proposed as clonal having only one mating type, whereas group B reproduces sexually, allowing the occurrence of recombination followed by segregation (Delye and Corio-Costet, 1998; Péros *et al.*, 2005; Corio-Costet, 2007; Montarry *et al.*, 2008; Montarry *et al.*, 2009). Also, most studies suggest that postzygotic barriers exist between the two genetic groups. Many researchers have failed to obtain viable progenies from the cross, and even in successful works (Stummer *et al.*, 2000; Stummer and Scott, 2003), most progenies had parental haplotypes with no random segregation between the markers. These aspects, therefore, may have significant implications for disease control practices, such as the type and duration of fungicide application and host resistance. Indeed, within the populations of *E. necator*, group A isolates have been reported as more sensitive to the different fungicides, while group B has been associated with fungicide resistance (Corio-Costet, 2007; Dufour *et al.*, 2011).

Knowledge of powdery mildew population diversity and structure may be useful to understand important aspects associated with this pathogen, such as biological cycle, host-parasite interactions, and disease control strategies (Glawe, 2008).

The high economic and environmental impact of powdery mildew on the wine industry demonstrates the importance of studying the Portuguese populations of *E. necator*. Two aspects, one associated with the host and another with the pathogen, may provide evidence for the existence of substantial genetic variation in the Portuguese populations of *E. necator*. Regarding the host, during the Quaternary glaciations that froze Northern and Central Europe, both the Iberian and the Italian Peninsulas seem to have played a particular role in *V. vinifera* speciation, acting as a warmer refuge where new varieties were developed. This may explain differentiation in wild populations before any domestication. Portuguese wild vine populations are

at the periphery of the species geographical distribution. Therefore, Portugal offers a unique richness in autochthonous cultivated varieties that contributes to the overall diversity of grapevine worldwide (Cunha *et al.*, 2010). Regarding the pathogen, the first case of *E. necator* azole resistance was observed in the same country (Steva *et al.*, 1989).

The main goal of this work was to describe the diversity and population structure in Portuguese isolates of *E. necator*.

MATERIALS AND METHODS

1. Sampling

The present study was conducted in the principal demarcated wine regions of Portugal: Alentejo (A), Bairrada (B), Dão (D), Douro (Dr), Setúbal (S), and

Vinhos Verdes (VV). In these regions, leaf and berry samples from powdery mildewed *V. vinifera* cultivars (spot symptoms) were collected, corresponding to a total of 280 samples (Table 1). Samples were individually placed in sealed plastic bags, frozen in the field using a portable freezer, and maintained at -20 °C until use.

A total of 56 samples, including infected leaves and berries collected from various cultivars grown in the main Portuguese demarcated wine regions, were included in this study. Among these samples, 10 were collected in Alentejo, 4 in Bairrada, 6 in Dão, 12 in Douro, 2 in Setúbal, and 22 in Vinhos Verdes.

2. DNA extraction and sequencing

The samples were brushed using a cytobrush to remove the fungal material (mycelium and spores)

Table 1 - Demarcated wine regions, location and *V. vinifera* cultivar of the samples included in this study.

Demarcated Wine Region (sampling date)	Location	Cultivars
Alentejo (18-22/Feb/2013)	Cuba	Alicante Bouschet (AB; red grapes)
	Évora	Aragonez (A; red grapes)
	Mértola	Chardonnay (C; white grapes)
	Pias	Trincadeira (T; red grapes)
	Moura	Not identified (X)
	Serpa	
Bairrada (7/Feb/2013)	Anadia	Chardonnay (C; white grapes) Fernão Pires (FP; white grapes)
Dão (4-5/Feb/2013)	Lousã	Cerceal (Cr; white grapes)
	Tondela	Cordões (Cd; red grapes)
	Vila Nova de Poiares	Not identified (X)
Douro (14-19/Feb/2013)	Murça	Cerceal (Cr; white grapes)
	Pinhão	Códega de Larinho (Co; white grapes)
	Régua	Gouveio (G; white grapes) Tinta Roriz (Tr; red grapes) Touriga Francesa (Tf; red grapes) Touriga Nacional (Tn; red grapes) Viosinho (V; white grapes)
Setúbal (6/Feb/2013)	Azeitão	Aragonez (A; red grapes)
Vinhos Verdes (5-13/Feb/2013)	Amarante	Alvarinho (Al; white grapes)
	Barcelos	Arinto (Ar; white grapes)
	Fafe	Azal branco (Ab; white grapes)
	Felgueiras	Borraçal (B; red grapes)
	Guimarães	Loureiro (L; white grapes)
	Melgaço	Malvasia (M; white grapes)
	Monção	Pedrenã (P; white grapes)
	Nogueira	Trajadura (Tj; white grapes)
	Roriz	Vinhão (Vi; red grapes)

Table 2 - Primers used for the identification of *E. necator*.

Gene	Primers	Sequence (5' → 3')	Annealing temperature (°C)	Fragment length (bp)	References
β-tubulin	Bt2c	CAGACTGGCCAATGCGTA	62	470	Brewer & Milgroom, 2010
	Bt2d	AGTTCAGCACCTCGGTGTA			
IGS*	IGS-12a	AGTCTGTGGATTAGTGGCCG	62	300	
	NS1R	GAGACAAGCATATGACTAC			
ITS**	ITSEnF	AAGGATCATTACAGAGCGAGAGG	62	480	
	ITSEnR	GGATGACCGGACAAAGGTG			

* the intergenic spacer region includes the 18S rRNA gene

** the internal transcribed spacer (ITS) includes the internal transcribed spacer 1, the 5.8S rRNA gene and the internal transcribed spacer 2 regions.

developing on the surface. Samples were suspended in water and submitted to mechanical disruption prior to extraction (Oliveira *et al.*, 2010). As previously described, a modified sodium hydroxide procedure was applied for DNA extraction (http://www.aspergillus.org.uk/indexhome.htm?secure/laboratory_protocols) (Oliveira *et al.*, 2014). Extracts were suspended in 50 µL of ultrapure water (Qiagen, Hilden, Germany) and stored at -20°C until further use.

Three genomic regions were used for the identification of *E. necator* populations. The first two genomic regions, previously identified in *E. necator*, included the internal transcribed spacer (ITS in Table 2) and intergenic spacer region (IGS in Table 2) of nuclear (Delye *et al.*, 1999; Brewer and Milgroom, 2010). Afterward, DNA extracts from both group A and group B, as defined from IGS and ITS sequencing data, were used for β-tubulin gene sequence analysis, for confirmation purposes (Brewer and Milgroom, 2010).

PCR amplification was conducted in a final volume of 5.0 µL, consisting of 2,5 µL of MyTaq™ HS MIX (Bioline), 1.0 µL of primer mix (each primer at 2.0 µM), 0.5 µL of fungal DNA (50-250 ng), and 1.0 µL of ultrapure water. PCR reactions were run at the following conditions: initial denaturation step at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 1 min and extension at 70°C for 1 min, followed by a final extension step at 70°C for 10 min. Amplification of PCR products was confirmed on a polyacrylamide gel and visualized by silver staining. Amplification products were purified using Exonuclease I (Thermo Scientific, Waltham, Massachusetts, USA) and FastAP (1:5) (Thermo Scientific). After purification, 2,5 µL of PCR purified product was added to 2,0 µL of sequencing kit (Applied Biosystems, Foster City, California, USA) and 0,5 µL of primer (5,0 µM). Sequencing reaction was run according to manufacturer instructions.

Samples were amplified using both forward and reverse primers. Sequencing data were processed and analyzed with Sequencing 5.2 analysis software (Applied Biosystems).

3. Sequence analysis

Sequences were aligned by using ClustalW (Thompson *et al.*, 1994); phylogenetic inference was made with the Maximum-Likelihood method using the Tamura-Nei model (Tamura and Nei, 1993); the branching patterns were validated by bootstrap based on 1000 resamplings, using MEGA6 software (Tamura *et al.*, 2013); and estimates of divergence and diversity and Tajima's Neutrality Test were calculated using MEGA6 software (Tamura *et al.*, 2013).

Thirteen ITS sequences of *E. necator* available in GenBank were included in this work: sequences AF011325 and GQ255473, representative of both genetic groups identified in the United States; AF049331 and AF049332, representative of both genetic groups identified in Europe; DQ189089 and AF073346, corresponding to group B samples from France and Australia, respectively; and the remaining sequences (AY818345, DQ444327, DQ841574, DQ872510, DQ899951, EF080860, and KJ539202) corresponding to group B samples from unknown provenance. Moreover, one IGS sequence (GQ255476) was included in this work, corresponding to a group A sample from the United States. The β-tubulin sequences used, AY074934 and GQ255475, are representative of both genetic groups identified in France and the United States.

RESULTS

DNA extraction was successfully conducted, allowing the management of plant material in less than 4 hours.

ITS and IGS regions were successfully amplified and sequenced. The alignment of all sequences from both

genomic regions revealed a total of four discriminative and informative polymorphisms: one clear polymorphism at position 70 in the ITS region where group A and B samples had a C and T nucleotide, respectively, and three clear polymorphisms at position 86, 194 and 201 in the IGS region where group A had C/C/T nucleotides and group B had T/T/C nucleotides (Table 3). All polymorphisms were found in introns. Subsequently, the corresponding phylogenetic trees were built and in both genomic regions the Portuguese population of *E. necator* was divided into two sub-populations. When the ITS region was considered, only four DNA samples (7.1 %) were assigned to the previously described group A of *E. necator*. Samples belonging to this group were identified in the Douro region (Murça), in two distinct white cultivars (Códega de Larinho and Gouveio) (Table 3). In group A were included the sequences AF049332 and DQ189089 corresponding to isolates from France and GQ255473 corresponding to isolates from the United States (all retrieved from GenBank). On the contrary, 52 DNA samples (92.9 %) were assigned to the previously described group B of *E. necator*. Samples belonging to this group were identified in all selected regions, in both white (Alvarinho, Azal Branco, Cerceal, Chardonnay, Fernão Pires, Loureiro, and Malvasia) and red grape cultivars (Aragonez, Borraçal, Cordões, Tinta Roriz, Touriga Francesa, Trincadeira, Vinhão, and Viosinho). In group B were included the sequences AF049331 from France, AF073346 from Australia, and AF011325 from the United States (all retrieved from GenBank). When the IGS region was considered, the same distribution was observed between the analyzed samples. The sequence GQ255476 retrieved from GenBank corresponds to group B (Table 3).

A set of 13 samples was then chosen to confirm the affiliation to groups A and B, by sequencing of the β -tubulin gene. According to the polymorphic nucleotide positions identified in this gene, the five samples identified as Pv26, TH03, En133 (from France), DrMrGC02 and DrMrCoC02 (from the Douro region, Portugal) belonged to group A, while the remaining eight samples belonged to group B (Figure 1).

All analyzed samples presented low genetic distance and low diversity, with the highest values observed using the IGS region corresponding to a genetic distance of 0.010 between groups A and B and a coefficient of differentiation of 0.972. These samples collected in different locations within the vineyard (on both leaves and berries) were compared and showed no variability among each plot. Moreover,

reference samples belonging to both groups A and B were included in the study (kindly provided by INRA-Bordeaux, France).

DISCUSSION

Grape powdery mildew, caused by the biotrophic fungus *E. necator* (formerly *Uncinula necator*; Ascomycota: Erysiphales), is the most widespread and destructive fungal disease of grapevine. Nowadays, and despite all preventive measures implemented, fungal diseases are still responsible for 20 to 40 % of productivity losses in Northern Portugal vineyards. Moreover, this pathogen is frequently associated with the production of low-quality wines due to the changes in sensory properties and composition.

As stated before, the previously reported protocol concerning the preparation, DNA extraction and amplification of *E. necator* samples is time consuming and almost impossible to implement to a large number of samples, making any attempt to block the destruction of a vineyard ineffective. Herein, a modified method was proposed for sample preparation and DNA extraction/amplification of *E. necator*.

Among the leaf and berry samples collected in the main Portuguese demarcated wine regions (Alentejo, Bairrada, Dão, Douro, Setúbal, and Vinhos Verdes), the calculated distance between DNA samples from groups A and B was 0.010. In these previous studies, the diversity of *E. necator* was revealed not only using IGS and ITS regions (Delye *et al.*, 1999; Brewer and Milgroom, 2010) but also β -tubulin (Amrani and Corio-Costet, 2006) and eburicol 14a-demethylase (CYP51) genes (Delye *et al.*, 1999), reaching similar conclusions. However, in the present study, the existence of the two groups was inferred using different genomic regions: ITS and IGS. These two gene fragments were able to separate the A and B groups of *E. necator*. ITS and IGS regions are commonly employed for fungal molecular identification and their amplification and sequencing analysis is technically less problematic than other gene analysis, facilitating the laboratory routine (Saenz *et al.*, 1994; Saenz and Taylor, 1999; Kovács *et al.*, 2011). The Portuguese population of *E. necator* was structured into two genetically differentiated groups (A and B), as previously identified in several grapevine-growing countries (Delye *et al.*, 1999; Stummer *et al.*, 2000; Péros *et al.*, 2005; Núñez *et al.*, 2006; Miazzi *et al.*, 2008; Montarry *et al.*, 2008; Brewer and Milgroom, 2010). However, it has been proposed that the frequencies of

Table 3. Informative nucleotide polymorphic positions in the *E. necator* samples included in the present study, including both reference strains and references retrieved from the NCBI database.

Samples marked with * correspond to database accession retrieved from the GenBank database, while samples marked with ** correspond to reference samples from group A (En133, PV26, and TH03) and B (En121, G1-01, G2-02, and PV02) kindly provided by INRA-Bordeaux.

Demarcated wine region	Sample reference	ITS		IGS	
		70	86	194	201
Alentejo	ACXC01 (Group B)	C	T	T	C
	ACXF01 (Group B)	C	T	T	C
	AMAC01 (Group B)	C	T	T	C
	AMAF01 (Group B)	C	T	T	C
	AMCC01 (Group B)	C	T	T	C
	AMCF01 (Group B)	C	T	T	C
	APAC01 (Group B)	C	T	T	C
	APAF01 (Group B)	C	T	T	C
	ASTC01 (Group B)	C	T	T	C
ASTF01 (Group B)	C	T	T	C	
Bairrada	BACC01 (Group B)	C	T	T	C
	BACF01 (Group B)	C	T	T	C
	BAFPC01 (Group B)	C	T	T	C
	BAFPF01 (Group B)	C	T	T	C
Dão	DLCrC01 (Group B)	C	T	T	C
	DLCrF01 (Group B)	C	T	T	C
	DPXC01 (Group B)	C	T	T	C
	DPXF01 (Group B)	C	T	T	C
	DTCdC02 (Group B)	C	T	T	C
DTCdF02 (Group B)	C	T	T	C	
Douro	DrMrCoC02 (Group A)	T	C	C	T
	DrMrCoF02 (Group A)	T	C	C	T
	DrMrGC02 (Group A)	T	C	C	T
	DrMrGF02 (Group A)	T	C	C	T
	DrMrVC02 (Group B)	C	T	T	C
	DrMrVF02 (Group B)	C	T	T	C
	DrPTfC02 (Group B)	C	T	T	C
	DrPTfF02 (Group B)	C	T	T	C
	DrRCC01 (Group B)	C	T	T	C
	DrRCF01 (Group B)	C	T	T	C
	DrRTC01 (Group B)	C	T	T	C
DrRTF01 (Group B)	C	T	T	C	
Setúbal	SAAC01 (Group B)	C	T	T	C
	SAAF01 (Group B)	C	T	T	C
Vinhos Verdes	VVAAbC01 (Group B)	C	T	T	C
	VVAAbF01 (Group B)	C	T	T	C
	VVAMC01 (Group B)	C	T	T	C
	VVAMF01 (Group B)	C	T	T	C
	VVBAC01 (Group B)	C	T	T	C
	VVBAF01 (Group B)	C	T	T	C
	VVBLC02 (Group B)	C	T	T	C
	VVBLF02 (Group B)	C	T	T	C
	VVCAC01 (Group B)	C	T	T	C
	VVCAF01 (Group B)	C	T	T	C
	VVFAC01 (Group B)	C	T	T	C
	VVFAF01 (Group B)	C	T	T	C
	VVFFPC01 (Group B)	C	T	T	C
	VVFFPF01 (Group B)	C	T	T	C
	VVMBC02 (Group B)	C	T	T	C
	VVMBF02 (Group B)	C	T	T	C
	VVNVC01 (Group B)	C	T	T	C
	VVNVF01 (Group B)	C	T	T	C
	VVPFC02 (Group B)	C	T	T	C
VVPPF02 (Group B)	C	T	T	C	
VVRLC02 (Group B)	C	T	T	C	
VVRLF02 (Group B)	C	T	T	C	

GenBank sequences	<i>E. necator</i> (AF011325)*	C	-	-	-
	<i>E. necator</i> (AF049331)*	C	-	-	-
	<i>E. necator</i> (AF049332)*	T	-	-	-
	<i>E. necator</i> (AF073346)*	C	-	-	-
	<i>E. necator</i> (AY818345)*	T	-	-	-
	<i>E. necator</i> (DQ444327)*	T	-	-	-
	<i>E. necator</i> (DQ189089)*	T	-	-	-
	<i>E. necator</i> (DQ841574)*	T	-	-	-
	<i>E. necator</i> (DQ872510)*	T	-	-	-
	<i>E. necator</i> (DQ899951)*	T	-	-	-
	<i>E. necator</i> (EF080860)*	T	-	-	-
	<i>E. necator</i> (GQ255473)*	T	-	-	-
	<i>E. necator</i> (KJ539202)*	T	-	-	-
	<i>E. necator</i> (GQ255476)*	-	C	C	T
	Reference strains (INRA-Bordeaux)	En121 (Group B)**	C	T	T
En133 (Group A)**		T	C	C	T
G1-01 (Group B)**		C	T	T	C
G2-02 (Group B)**		C	T	T	C
Pv02 (Group B)**		C	T	T	C
PV26 (Group A)**		T	C	C	T
TH03 (Group A)**		T	C	C	T

these two groups vary considerably among vineyards. The majority of the samples included in the present work belonged to group B (92.6 %), whereas only four samples, corresponding to samples collected from leaves and berries of Códéga de Larinho and Gouveio cultivars from Murça in the Douro region, were assigned to group A. These two white grape cultivars are known to present different susceptibility to this fungus: Gouveio has been considered susceptible, whereas Códéga de Larinho is less sensitive (Sousa *et al.*, 2007). These two *E. necator* samples were collected in the same vineyard being absent in the surrounding locations in the Douro region, such as Peso da Régua and Pinhão, indicating a possible pattern of spatial segregation among *E. necator* groups. Spatial distribution demonstrated high heterogeneity between vineyards: from 100 % A to 100 % B; however, the majority of the fields presented variable presence of both isolates (Montarry *et al.*, 2008). Temporal isolation appears to be the primary mechanism to maintain the highly differentiated genetic structure in *E. necator* populations (Corio-Costet *et al.*, 2000; Amrani and Corio-Costet, 2006; Corio-Costet, 2007; Montarry *et al.*, 2009). In fact, it has been proposed that despite the initial frequency of both groups, towards the end of the epidemics, group A isolates, acquired at the beginning of the growing season, tend to disappear over the course of the epidemics, whereas group B isolates remain active, being the only group active by the end of the epidemics (Montarry *et al.*, 2008). This was the case of the samples included in the present study, as the sampling period (post-harvest season) can explain the vast majority of the samples assigned to group B. This temporal pattern may result in differences in aggressiveness (i.e., quantitative

pathogenicity): an initial attack by a group B population causes more damage at harvest, while if group B isolates increase in frequency later (when ontogenic or age-related resistance is active), little or no damage is observed during harvest (Montarry *et al.*, 2009). Isolates assigned to group A, genetically less diverse, have been suggested to be clonal, whereas group B is sexually reproducing, allowing the occurrence of recombination followed by segregation (Péros *et al.*, 2005). However, the production of a fertile and viable ascospore progeny is still under debate (Miazzi *et al.*, 2003; Stummer and Scott, 2003).

As a conclusion, the present work allowed the molecular characterization of *E. necator* strains collected from diverse *V. vinifera* cultivars in the main Portuguese demarcated wine regions. ITS and IGS regions were successfully amplified and made it possible to obtain a good resolution in terms of separation between *E. necator* groups A and B. The majority of the samples analyzed belonged to group B and only four samples collected in the Douro region belonged to group A. The affiliation of the Portuguese samples to these previously described groups was further confirmed by sequencing of the β -tubulin gene in some of the samples.

The results point to new directions that could be further explored. From the point of view of molecular-based diagnostic, a SNaPshot protocol could be developed. After the selection of the indicative polymorphic positions, instead of performing the regular protocol that includes the amplification, sequencing and analysis of seven genomic regions, a single minisequencing protocol could be applied, providing the same amount of

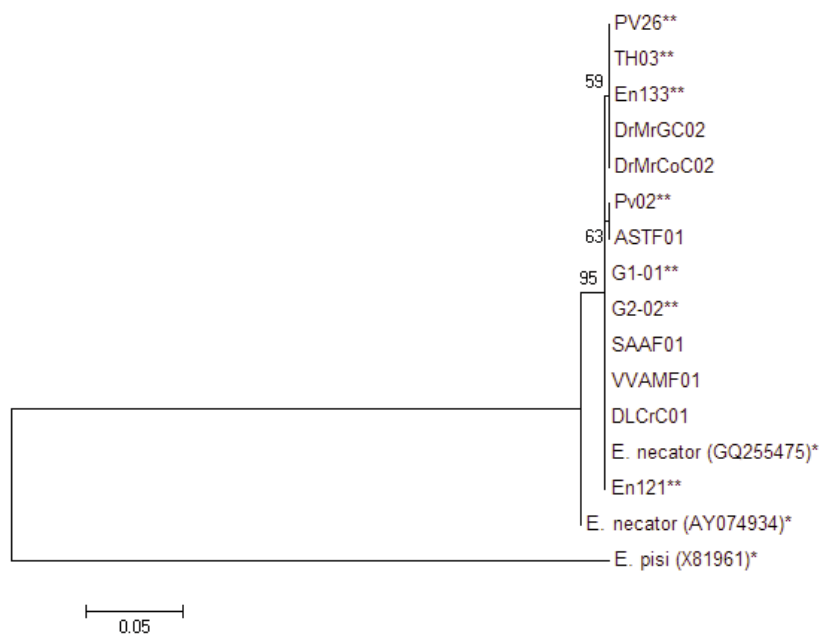


Figure 1. Molecular phylogenetic analysis based on the β -tubulin region of 16 nucleotide sequences.

The evolutionary history was inferred by using the Maximum-Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1161.3141) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 495 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). Samples marked with * correspond to database accession retrieved from NCBI GenBank, while samples marked with ** correspond to reference samples from group A (En133, PV26, and TH03) and B (En121, G1-01, G2-02, and PV02) kindly provided by INRA-Bordeaux. *E. pisi* served as an outer group for phylogenetic reconstruction.

information with a reliable, rapid and cost-effective procedure. From the point of view of diversity, it would be interesting to compare the yearly fluctuations in the prevalence of groups A and B among different years and to try to correlate these changes with the incidence and the intensity of fungal disease attacks.

The pathogen *E. necator* has a significant environmental, economic and biological impact on the protection and production of grape products. The present work will certainly help to increase the productivity of the grape products through a better definition of preventive measures and a potential decrease in the direct and indirect costs associated with the use of fungicides.

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