Pertanika J. Trop. Agric. Sci. 35 (S): 79 - 84 (2012)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Molecular Identification and Phylogenetic Analysis of *Pseudoperonospora cubensis* Isolates in Peninsula Malaysia

Salati, M.[1], Sariah, M.[1,2], Tan, Y. H.[1] and Wong, M.[1,2*]

[1]*Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.*

[2]*Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Thirteen isolates of *Pseudoperonospora cubensis*, the causal agent of downy mildew, were collected from cucurbit fields in five states of the western part of Peninsular Malaysia during its growing season between November 2008 and March 2009. The host range of these isolates was determined previously using leaf disc assay and the results indicated that there were 12 pathotypes among these isolates. The objective of this study was to analyze the 13 isolates for phylogenetic relationship using internal transcribed spacers (ITS) of ribosomal DNA (rDNA) and mitochondrial COX-II regions. A high sequence similarity among the 13 isolates and similar sequences from GenBank were detected in ITS (>99%) and COX-II (>98%) regions. Phylogenetic analysis of the 13 isolates based on Minimum Evolution method performed on ITS and COX-II regions revealed five and three groupings, respectively. However, no relationship was found between the phylogenetic groupings using both genes and pathotypes in this study.

Keywords: Pathotype, genetic diversity, cucumber downy mildew

INTRODUCTION

Downy mildew is one of the more common diseases of cucurbits, caused by the obligate biotrophic oomycete, *Pseudoperonospora cubensis* (Berk. et Curt.) Rostow. Recently in Europe and the United States, a severe infection by *P. cubensis* in cucumber plants was reported and the pathogen was demonstrated to have high variability in pathogenicity over 60 cucurbit species (Lebeda & Cohen, 2011). The importance of internal transcribed spacers (ITS) sequence

ARTICLE INFO

Article history:
Received: 18 June 2012
Accepted: 19 October 2012

E-mail addresses:
salati1@yahoo.com (Salati, M.),
sariahm@putra.upm.edu.my (Sariah, M.)
alvinyht@agri.upm.edu.my(Tan, Y. H.)
muiyun@putra.upm.edu.my (Wong, M.)
*Corresponding author

ISSN: 1511-3701

analysis of ribosomal DNA (rDNA) and mitochondrial encoded cytochrome c oxidase 2 (COX-II) genes have received increasing attention for their role in evaluating the taxonomic and phylogenetic relationships of species with different degrees of intraspecific diversity (Choi *et al.*, 2006).

To date, there has been little information on intraspecific molecular variation among the *P. cubensis* samples collected from the different areas in Peninsular Malaysia. The aims of this study were to examine the genetic diversity of *P. cubensis* isolates belonging to different pathotypes based on the ITS and COX-II polymorphic regions and to determine which of these two regions could be applied to differentiate isolates belonging to the various pathotypes. A total of 13 isolates were collected from five states of Peninsular Malaysia and their pathotypes were identified previously using the leaf disc method (Salati *et al.*, 2010).

MATERIALS AND METHODS

The isolates of *P. cubensis* identified and characterized previously (Salati *et al.*, 2010) were used for the phylogenetic study. The extraction of the total genomic DNA from leaves dried in silica gel was conducted based on the modified CTAB method. As for the amplification of complete ITS1-5.8S-ITS2 and COX-II regions, the universal primers, ITS5-P2/ITS4 (Voglmayr and Constantinescu, 2008) and COX-II specific primers (Hudspeth *et al*., 2000) were respectively used. The amplified regions were directly sequenced at both strands using an automatic sequencer. DNA sequences were analyzed for similarity using the basic logical alignment search tool (BLAST), while multiple sequence alignment was performed using the CLUSTAL W2 programme. The evolutionary history inferred using the Minimum Evolution method available in MEGA4 (Version 4.0.2) software.

RESULTS AND DISCUSSION

Fragments of 802 bp were obtained from all 13 isolates, whose ITS region was studied (Table 1) and divided into three, namely, ITS1 (1-216 bp), 5.8S rDNA (217-378 bp) and ITS2 (379-802 bp). The length of the ITS sequences is consistent with the finding of Choi *et al.* (2005) from Korea and Sarris *et al.* (2009) from Greece and the Czech Republic. The results of the BLAST analysis comparing ITS sequences in GenBank confirmed that the detected pathogen was *Pseudoperonospora cubensis.* The greatest variations in the whole ITS1-5.8S-ITS2 sequence was observed in ITS2 region (1.9%), followed by ITS1 (1.3%), similar to that found in a study using *Albugo candida* isolates (Choi *et al.*, 2006). All 5.8S region of the 13 isolates studied did not show any polymorphism in nucleotide which could be considered a conserved region. In the phylogenetic analysis, the optimal tree with a branch length sum of 0.04092469 was calculated for the full length of ITS regions (Fig.1). The result revealed five groupings (A, B, C, D and E) among the 13 isolates. Isolates EU660054 (Voglmayr *et al*., 2009), AY608618 (Choi *et al*.,

TABLE 1

GenBank accession numbers of internal transcribed spacers (ITS) and mitochondrial encoded cytochrome c oxidase 2 (COX-II) regions of *P. cubensis* isolates

Isolate	Length (bp)		Accession number in GenBank	
	ITS	COX-II	ITS	COX-II
A1	802	556	HM208310	HM988994
A7	802	556	HM208311	HM988995
A9	802	559	HM208312	HM988996
A10	802	556	HM208313	HM988997
B1	802	557	HM208314	HM988998
B2	802	553	HM208315	HM988999
C1	802	556	HM208316	HM989000
C2	802	556	HM208317	HM989001
C4	802	556	HM208318	HM989002
D1	802	556	HM208319	HM989003
D2	802	556	GU233293	HM989004
D3	802	556	HM208320	HM989005
E1	802	555	HM208321	HM989006

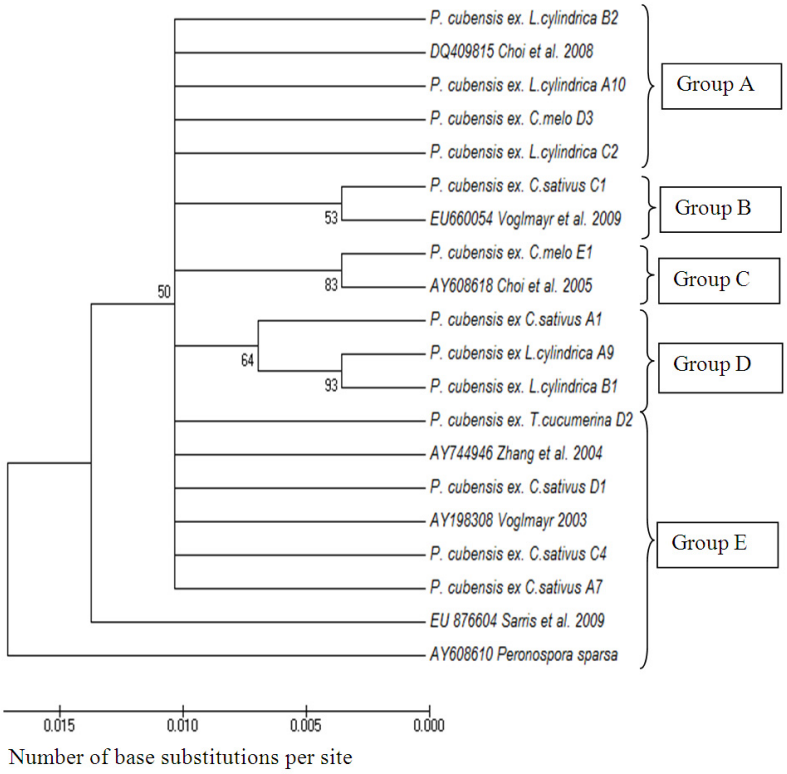

Fig.1: Phylogenetic tree based on the sequencing results on ITS regions of *P. cubensis* isolates (A1, A7, A9, A10, B1, B2, C1, C2, C4, D1, D2, D3 and E1) in comparison with the reference isolates. *Peronospora sparsa* is an out group isolate. The bootstrap number more than 50 was selected in Minimum Evolution tree

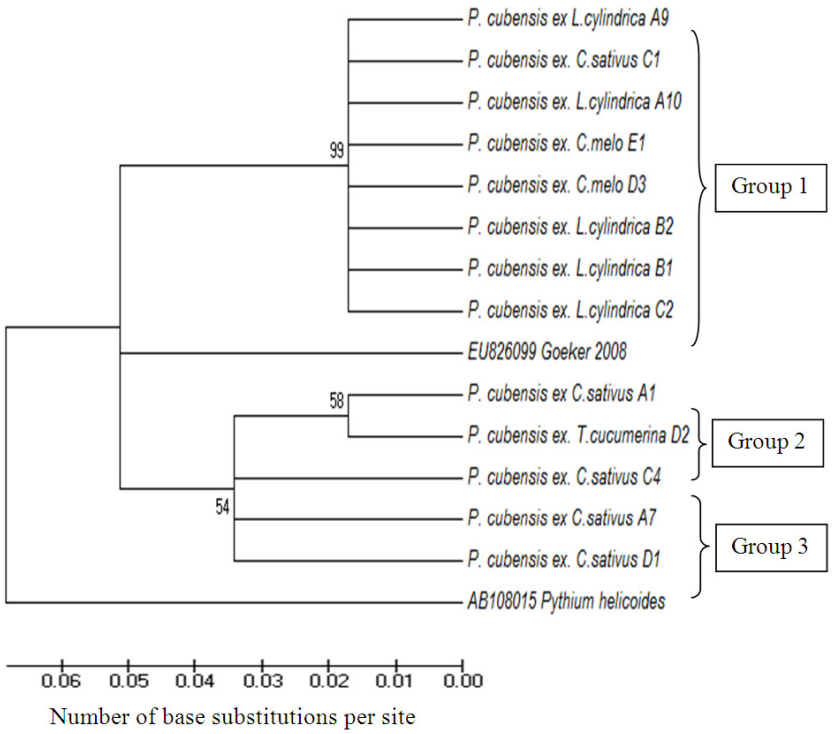

Fig.2: Phylogenetic tree based on the sequencing results on COX-II region of *P. cubensis* isolates (A1, A7, A9, A10, B1, B2, C1, C2, C4, D1, D2, D3 and E1) in comparison with the reference isolate, EU826099, obtained from GenBank. *Pythium helicoides* was selected as an out group species. The bootstrap value more than 50 was selected in Minimum Evolution tree

2005), AY744946 (Zhang *et al*., 2004) and AY198308 (Voglmayr, 2003) from various geographical regions used as comparisons were placed within the five groupings, whereas isolate EU876604 (Sarris *et al*., 2009) placed in a separate branch and *Peronospora sparsa* (AY608610) was shown to be an outgroup. The minimum sequences similarity within the five groupings was detected at 99.5%, 99.5%, 100%, 99.7% and 99.9%, respectively. Two isolates, with the same pathogenic factor and pathotype number (namely, D1 and D2), were placed together in one group (E). In the present study, the ITS sequence homology among the isolates from various hosts was very high similar with that of Choi *et al.* (2005), suggesting that *P. cubensis* is a homogenous taxon.

The COX-II region was amplified from all the 13 isolates with sequences ranging from 553-559 bp (Table 1). Results of the BLAST analysis showed that all the 13 isolates shared 98-99% similarity with *P. cubensis* COX-II sequences in the GenBank. In the phylogenetic analysis, the optimal tree with a branch length sum of 0.14932510 was calculated for COX-II region (see Fig.2). All the 13 isolates were delineated into three groupings (1, 2 and 3), where isolate EU826099 (Goeker, 2008) used as a comparison was placed in

a separate branch. Meanwhile, *Pythium helicoides* (AB108015) was shown to be an outgroup. The minimum sequences similarity within the first, second and third grouping was detected at 99.8%, 99.6% and 100%, respectively. The similarity among the isolates based on the COX-II region was higher than those based on the ITS region indicated by lesser groupings. In contrast to the phylogeny based on the ITS region, two isolates (D1 and D2) with the same pathogenic and pathotype number were placed in different groups of one cluster.

The results of the current study indicated that nuclear locus, demonstrated by the ITS phylogram (five groupings), was more diverse than mitochondria locus, as shown by COX-II phylogram (three groupings) and these findings corroborate with those of Choi *et al.* (2006) and of *Phytophthora capsici* (Quesada-Ocampo *et al*., 2011). Interestingly, the results of the present study also showed that there was no correlation between the genetic clusters of both nuclear and mitochondria loci and the pathotypes of the isolates studied (Salati *et al.*, 2010) similar to that reported of *Phytophthora capsici* (Quesada-Ocampo *et al*., 2011). As a conclusion, the present study demonstrated that the utilization of ITS and COX-II regions was useful for the identification of *P. cubensis* at species level but was rather inadequate for the differentiation of *P. cubensis* pathotypes. Thus, the information suggests that multi-locus analyses and the association of virulence/pathogenicity with particular clusters should be used to obtain higher phylogenetic resolution among the isolates from Peninsula Malaysia and for host resistance screenings (Runge *et al*., 2011; Quesada-Ocampo *et al*., 2011).

ACKNOWLEDGEMENTS

The authors wish to thank the Ministry of Higher Education, Malaysia, for the partial sponsorship of the present study through Fundamental Research Grant Scheme (FRGS).

REFERENCES

Choi, Y. J., Hong, S. B., & Shin, H. D. (2005) A re-consideration of *Pseudoperonospora cubensis* and *P. humuli* based on molecular and morphological data. *Mycol Res., 109*, 841-848.

Choi, Y. J., Hong, S. B., & Shin, H. D. (2006) Genetic diversity within the *Albugo candida* complex (Peronosporales, Oomycota) inferred from phylogenetic analysis of ITS rDNA and COX2 mtDNA sequences. *Mol Phylogenet Evol., 40,* 400-409.

Hudspeth, D. S. S., Nadler, S. A., & Hudspeth, M. E. S. (2000). A COX2 molecular phylogeny of the Peronosporomycetes. *Mycologia, 92,* 674-684.

Lebeda, A., & Cohen, Y. (2011). Cucurbit downy mildew (*Pseudoperonospora cubensis*)—biology, ecology, epidemiology, host-pathogen interaction and control. *Eur. J. Plant Pathol., 129,* 157-192.

Lebeda, A., & Widrlechner, M. P. (2003). A set of Cucurbitaceae taxa for differentiation of *Pseudoperonospora cubensis* pathotypes. *J. Plant Dis. Protect., 110,* 337-349.

Quesada-Ocampo, L. M., Granke, L. L., Mercier, M. R., Olsen, J., & Hausbeck, M. K. (2011) Investigating the genetic structure of Phytophthora capsici populations. *Phytopathology, 101*, 1061-1073.

Runge, F., Choi, Y. J., & Thines, M. (2011). Phylogenetic investigations in the genus *Pseudoperonospora* reveal overlooked species and cryptic diversity in the *P. cubensis* species cluster. *Eur J Plant Pathol.*, *129,* 135-146.

Salati, M., Wong, M. Y., Sariah, M., & Nik, M. H. (2010). Host range evaluation and morphological characterization of *Pseudoperonospora cubensis*, the causal agent of cucurbit downy mildew in Malaysia. *Afr. J. Biotechnol., 9,* 4897-4903.

Sarris, P. F., Abdelhalim, M., Kitner, M., Skandalis, N., Panopoulos, N., Doulis, A. G., & Lebeda, A. (2009). Molecular polymorphism between populations of *Pseudoperonospora cubensis* from Greece and the Czech Republic and their phytopathological and phylogenetic implications. *Plant Pathol., 58*, 933-943.

Voglmayr, H., & Constantinescu, O. (2008). Revision and reclassification of three *Plasmopara* species based on morphological and molecular phylogenetic data. *Mycol. Res., 112*, 487-501.