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ORIGINAL ARTICLE

Peronosclerospora australiensis is a synonym of *P. maydis*, which is widespread on Sumatra, and distinct from the most prevalent Java maize downy mildew pathogen

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Abstract

This study was performed to identify *Peronosclerospora* species found in Indonesia based on sequence analysis of the *cox*2 gene. In addition, sequence data in total, 26 isolates of *Peronosclerospora* were investigated in this study. They were obtained from 7 provinces in Indonesia, namely Lampung, Jawa Timur, Jawa Barat, Sumatera Utara, Jawa Tengah, Yogyakarta, and Sulawesi Selatan. Sequence analysis of *cox*2 and phylogenetic inference were performed on all the 26 isolates. A set of primers developed in this study, PCOX2F and PCOX2R, was used for PCR amplification. Phylogenetic analyses showed that all the Indonesian isolates were divided into two groups. Group I contained 13 isolates; 9 isolates obtained from Lampung, 3 isolates from Sumatera Utara, and 1 isolate from Jawa Barat. Group II consisted of 13 isolates; 7 isolates from Jawa Timur, 2 isolates from Jawa Tengah, 1 isolate from Yogyakarta, and 3 isolates from Sulawesi Selatan. All the members of group I clustered with the ex-type sequence of *P. australiensis*. Meanwhile, all members of Group II formed the sister clade of isolates obtained from Timor-Leste and may represent *P. maydis*.

Keywords Biosecurity · *cox*2 · Genetic variation · Graminicolous downy mildews · Phylogeny

Introduction

Downy mildew of *Zea mays* (maize, corn) caused by *Peronosclerospora* spp. is one of the most important diseases in this crop, causing severe economic losses worldwide (Bonde 1982; Singh et al. 1987; Telle et al. 2011). Eleven species of *Peronosclerospora* have been reported until a decade ago, namely *P. dichanthiicola*, *P. eriochloae*, *P. heteropogonis*, *P. maydis*, *P. miscanthi*, *P. noblei*, *P. philippinensis*, *P. sacchari*, *P. sorghi*, *P. spontanea*, and *P. westonii*. In 2012, Shivas et al. (2012) described two new species of *Peronosclerospora*, namely *P. australiensis* and *P. sargae*. Three species have been reported as the causal agent of downy mildew on maize in Indonesia namely *P. maydis*, *P. philippinensis*, and *P. sorghi* (Bonde 1982; Muis et al. 2013; Rustiani et al. 2015; Muis et al. 2016). Interestingly, Telle et al. (2011) found that maize is infected by *P. eriochloae* and Shivas et al. (2012) revealed that their newly described species, *P. australiensis*, infects both native grasses and maize.

Species of *Peronosclerospora* in Indonesia have mainly been identified on the basis of host identity and morphological characteristics (Hikmahwati et al. 2011; Widiantini et al. 2015; Muis et al. 2016). This approach has limitations, and may cause unreliable identification results, as conidia, which serve as a key structure for identification, are very similar between some species (Telle et al. 2011). In addition, it has been reported for other downy mildews that conidal dimensions may vary depending on host, organ affected, and humidity (Runge and Thines 2011, Delanoe 1972, Dudka et al.

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2007, respectively). Moreover, for most species affecting maize, oospores that often provide a more stable means of identification are not known to occur frequently or at all in the species *P. maydis*, *P. philippinensis*, and *P. sorghi*, which affect maize crop in Indonesia. Identification is additionally complicated by the possibility that some species might have been described more than once, but type specimens or authentic material are difficult to investigate due to the evanescent nature of the conidiophores (Spencer and Dick 2002) and the often rapid deterioration of DNA in tropical specimens.

In order to confirm species identity of *Peronosclerospora* inferred on the basis of morphological characteristics, sequence analysis is essential (Telle et al. 2011; Shivas et al. 2012). Sequence analysis of Cytochrome C Oxidase subunit II (*cox*2) has been widely performed to identify species of oomycetes, including *Peronosclerospora* (Telle et al. 2011; Thines et al. 2015; Choi et al. 2015). Using sequence analysis of partial *cox*2 and nrLSU (nuclear ribosomal large subunit), Telle et al. (2011) could distinguish groups of Australian *Peronosclerospora* species and confirmed the absence of *P. maydis* from the continent. It was the aim of this study to investigate the identity of *Peronosclerospora* species found in Indonesia, including sequence analysis of the *cox*2 gene.

Materials and methods

Specimens of *Peronosclerospora*

For DNA extraction, samples of infected maize plants with downy mildew symptoms were collected throughout Indonesia. In total, 27 isolates of *Peronosclerospora* from 7 provinces in Indonesia were analyzed in this study (Table 1). Twenty-three specimens (CX1 – 3, KM, PRS, MK2, KD3SG, KD4SG, MDR, NGK, CK, MC, TR, DS, BY, KL, Y, ER5, L2, P2, TGM, BJR and BJS) were received as infected leaves and for 3 isolates (Ppr, Smbh, Jnu) *cox*2 sequences were obtained directly from DNA extracts, the type specimen has been obtained from KRAM (Table 1). DNA of most specimens has been deposited in the Herbarium Senckenbergianum (FR) under the accession number B001-1 to B001-23 (Table 1).

Molecular identification

DNA extraction The infected leaves of fresh specimens were cut to pieces (1 cm^2) and 10 pieces per sample were placed into a cold mortar. For the grinding of the material, 500 μl extraction buffer (1 M Tris-HCL (tris (hydroxmethyl) aminomethane-HCL) pH 8.0, 10% SDS, 5 M NaCl (Natrium chloride), 2% CTAB (cetyltrimethyl ammonium bromide)) was added into the mortar and the leaves were disrupted using a grinding pestle. After the sample was ground to a suspension, the mortar was covered using aluminum foil and incubated at − 40 °C overnight. Afterwards, before melting, the plant material was ground again until it became suspension. In total, 600 μl of the suspension was transferred to a 1.5 mL tube, 400 μl of 2% CTAB was added, and the tube incubated at 65 °C for 1 h. After incubation, 500 μl phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the tubes vigorously shaken by hand. Subsequently, tubes were centrifuged at 14,000 rpm in a microcentrifuge (Microspin12, Biosan, Latvia) for 10 min. The supernatant was transferred to a new 1.5 mL tube and chloroform/isoamyl alcohol (24:1) was added with the same volume as the supernatant, the tube shaken by hand, and then centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to a new 1.5 mL tube, and cold isopropanol (60% of volume) was added. After this, tubes were vigorously mixed by hand and incubated at − 40 °C for 10 min and then centrifuged at 14,000 rpm for 10 min. The supernatant was removed and 500 μl cold ethanol 70% was added into the tube. After this, it was centrifuged at 14,000 rpm for 5 min. The supernatant was removed and the pellet air-dried overnight. After this, 30–50 μl 1× TE buffer (pH 8.0) (1st Base, Malaysia) was added. DNA extraction from the type specimen of *P. maydis* was done as described in Telle et al. (2008) and also PCR was done according to that publication.

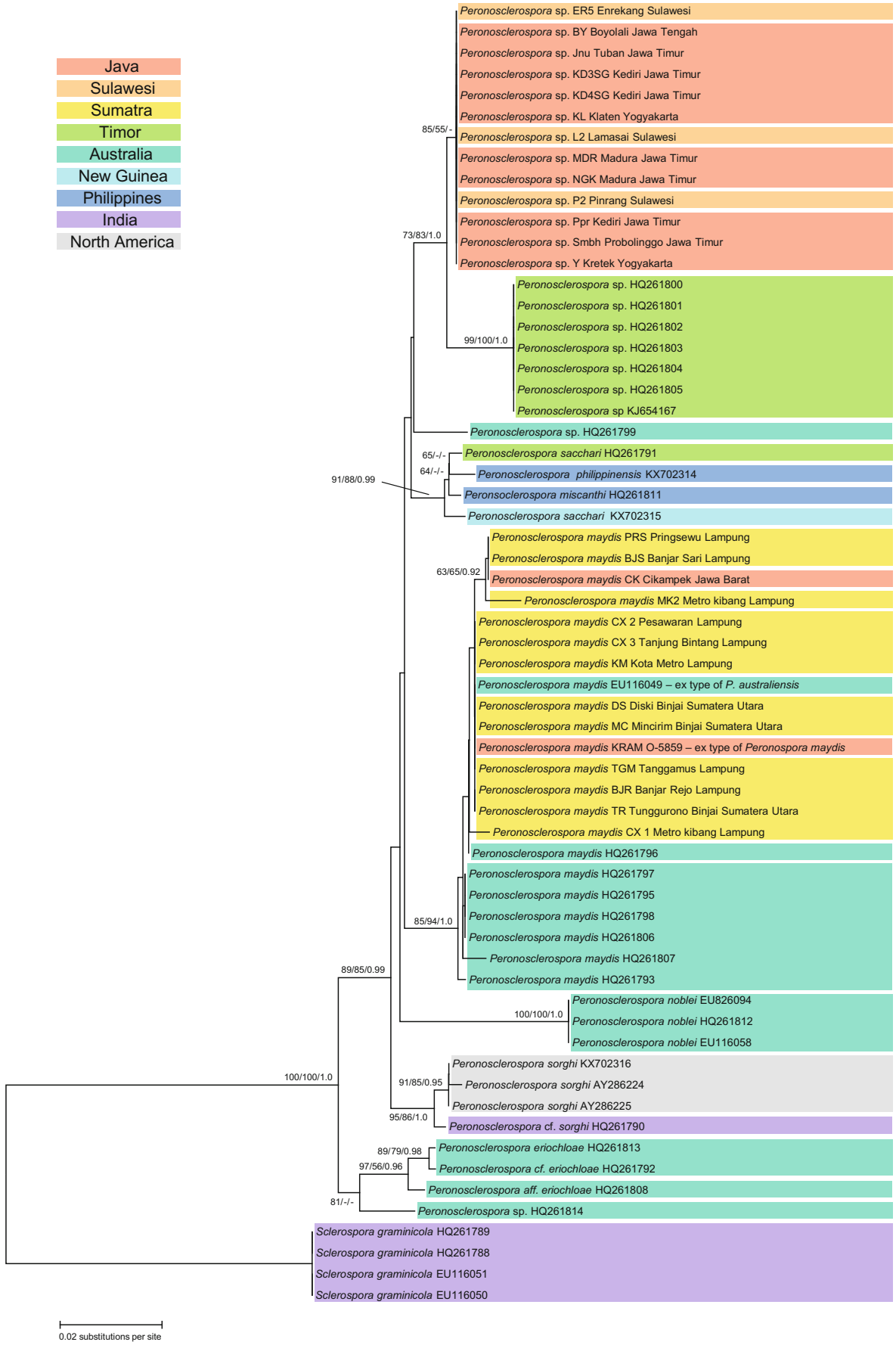
PCR amplification PCR was performed using a thermal cycler (Sensoquest Thermal Cycler, Sensoquest, Germany). In this study, a set of primer was developed from available oomycete sequences of cytochrome C oxidase subunit II (*cox*2) that were used for PCR amplification, namely PCOX2F (TCCAGCAA CTCCAGTTATGG) and PCOX2R (ACCTGGACAAGCAT CTAATT). This primer pair produces an amplicon of 529 bp. PCR was performed using MyTaq™ HS RedMix (Bioline, USA) PCR Kit according to the instructions of the manufacturer. As for DNA amplification, initial denaturation was carried out at 95 °C for 5 min, continued with 30 cycles of denaturation at 95 °C for 1 min; primer annealing at 48–52 °C for 1 min, primer extension at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. PCR products were evaluated using a DigiDoc UV trans illuminator (UVP, USA) after electrophoresis through an 0.5% agarose gel (*w*/*v* using 1× Tris Boric Acid EDTA (TBE) pH 8.0, 1st Base, Malaysia), containing 1 μL ethidium bromide (1 μg/mL) at 50 V for 70 min.

Sequencing and sequence analyses The PCR product from the *cox*2 amplification was sent to 1st Base Malaysia for bi-directional sequencing, using the primers used in PCR. The resulting chromotograms were edited using BioEdit for windows ver 7.2.6 (Hall 1999). Reference sequences used in this study were those from Telle et al. 2011, with some additional sequences obtained from NCBI using the TrEase webserver (Mishra et al. (unpublished), http://www.thines-lab.senckenberg.de/trease) with standard settings. Alignments were generated using muscle (Edgar 2004) as implemented on the TrEase webserver. Leading

Table 1 *Peronosclerospora* isolates from *Zea mays* used in this study

Isolate name	Island	Province	County	Year	GenBank accession (*cox*2)	Herbarium accession number for DNA extracts
CX-1	Sumatra	Lampung	Metro Kibang	2015	MT506921	DNA used up
CX-2	Sumatra	Lampung	Negeri Sakti	2015	MT506920	DNA used up
CX-3	Sumatra	Lampung	Tanjung Bintang	2015	MT506919	DNA used up
Ppr	Java	Jawa Timur	Kediri	2016	MT506927	DNA used up
Smbh	Java	Jawa Timur	Probolinggo	2016	MT506926	DNA used up
Jnu	Java	Jawa Timur	Tuban	2016	MT506935	DNA used up
KM	Sumatra	Lampung	Kota Metro	2016	MT506917	FR B001-1
PRS	Sumatra	Lampung	Pringsewu	2016	MT506914	FR B001-2
MK2	Sumatra	Lampung	Metro Kibang	2016	MT506915	FR B001-3
KD3SG	Java	Jawa Timur	Kediri	2016	MT506934	FR B001-4
KD4SG	Java	Jawa Timur	Kediri	2016	MT506933	FR B001-5
MDR	Java	Jawa Timur	Madura	2016	MT506930	FR B001-6
NGK	Java	Jawa Timur	Madura	2016	MT506929	FR B001-7
CK	Java	Jawa Barat	Cikampek	2016	MT506922	FR B001-8
MC	Sumatra	Sumatera Utara	Mincirim	2017	MT506916	FR B001-9
TR	Sumatra	Sumatera Utara	Tunggurono	2017	MT506912	FR B001-10
DS	Sumatra	Sumatera Utara	Diski	2017	MT506918	FR B001-11
BY	Java	Jawa Tengah	Boyolali	2017	MT506937	FR B001-12
KL	Java	Jawa Tengah	Klaten	2017	MT506932	FR B001-13
Y	Java	Yogyakarta	Bantul	2017	MT506925	FR B001-14
ER5	Sulawesi	Sulawesi Selatan	Enrekang	2017	MT506936	FR B001-15
L2	Sulawesi	Sulawesi Selatan	Lamasai	2017	MT506931	FR B001-16
P2	Sulawesi	Sulawesi Selatan	Pinrang	2017	MT506928	FR B001-17
TGM	Sumatra	Lampung	Tanggamus	2018	MT506913	FR B001-18
BJR	Sumatra	Lampung	Banjar Rejo	2019	MT506924	FR B001-19
BJS	Sumatra	Lampung	Banjar Sari	2019	MT506923	FR B001-20
TYPE	Java	Jawa Tengah	Tegal	s.d	MW025835	KRAM O-5859(J)

and trailing gaps were removed from the final alignment, which did not contain internal gaps. Phylogenetic inference for Minimum Evolution was done using MEGA7 (Kumar et al. 2016) using the Tamura-Nei substitution model and 1000 bootstrap replicates. Maxiumum likelihood analysis was done using RAxML (Stamatakis 2014) as implemented on the TrEase webserver, using the GTRGAMMAI substitution model. Bayesian inference was done using MrBayes (Ronquist et al. 2012) as implemented on the TrEase webserver, running 5 million generations and discarding the first 30% of the sampled trees for ensuring a sampling from the stationary phase. The resulting trees were visualized and edited using MEGA7. Sequences were submitted to GenBank and the corresponding accession numbers are given in Table 1.


Java
Sulawesi
Sumatra
Timor
Australia
New Guinea
Philippines
India
North America
Peronosclerospora sp. ER5 Enrekang Sulawesi
Peronosclerospora sp. BY Boyolali Jawa Tengah
Peronosclerospora sp. Jnu Tuban Jawa Timur
Peronosclerospora sp. KD3SG Kediri Jawa Timur
Peronosclerospora sp. KD4SG Kediri Jawa Timur
Peronosclerospora sp. KL Klaten Yogyakarta
Peronosclerospora sp. L2 Lamasai Sulawesi
Peronosclerospora sp. MDR Madura Jawa Timur
Peronosclerospora sp. NGK Madura Jawa Timur
Peronosclerospora sp. P2 Pinrang Sulawesi
Peronosclerospora sp. Ppr Kediri Jawa Timur
Peronosclerospora sp. Smbh Probolinggo Jawa Timur
Peronosclerospora sp. Y Kretek Yogyakarta
Peronosclerospora sp. HQ261800
Peronosclerospora sp. HQ261801
Peronosclerospora sp. HQ261802
Peronosclerospora sp. HQ261803
Peronosclerospora sp. HQ261804
Peronosclerospora sp. HQ261805
Peronosclerospora sp KJ654167
Peronosclerospora sp. HQ261799
Peronosclerospora sacchari HQ261791
Peronosclerospora philippinensis KX702314
Peronsoclerospora miscanthi HQ261811
Peronosclerospora sacchari KX702315
Peronosclerospora maydis PRS Pringsewu Lampung
Peronosclerospora maydis BJS Banjar Sari Lampung
Peronosclerospora maydis CK Cikampek Jawa Barat
Peronosclerospora maydis MK2 Metro kibang Lampung
Peronosclerospora maydis CX 2 Pesawaran Lampung
Peronosclerospora maydis CX 3 Tanjung Bintang Lampung
Peronosclerospora maydis KM Kota Metro Lampung
Peronosclerospora maydis EU116049 – ex type of P. australiensis
Peronosclerospora maydis DS Diski Binjai Sumatera Utara
Peronosclerospora maydis MC Mincirim Binjai Sumatera Utara
Peronosclerospora maydis KRAM O-5859 – ex type of Peronospora maydis
Peronosclerospora maydis TGM Tanggamus Lampung
Peronosclerospora maydis BJR Banjar Rejo Lampung
Peronosclerospora maydis TR Tunggurono Binjai Sumatera Utara
Peronosclerospora maydis CX 1 Metro kibang Lampung
Peronosclerospora maydis HQ261796
Peronosclerospora maydis HQ261797
Peronosclerospora maydis HQ261795
Peronosclerospora maydis HQ261798
Peronosclerospora maydis HQ261806
Peronosclerospora maydis HQ261807
Peronosclerospora maydis HQ261793
Peronosclerospora noblei EU826094
Peronosclerospora noblei HQ261812
Peronosclerospora noblei EU116058
Peronosclerospora sorghi KX702316
Peronosclerospora sorghi AY286224
Peronosclerospora sorghi AY286225
Peronosclerospora cf. sorghi HQ261790
Peronosclerospora eriochloae HQ261813
Peronosclerospora cf. eriochloae HQ261792
Peronosclerospora aff. eriochloae HQ261808
Peronosclerospora sp. HQ261814
Sclerospora graminicola HQ261789
Sclerospora graminicola HQ261788
Sclerospora graminicola EU116051
Sclerospora graminicola EU116050
85/55/-
73/83/1.0
99/100/1.0
65/-/-
64/-/-
91/88/0.99
63/65/0.92
85/94/1.0
89/85/0.99
100/100/1.0
100/100/1.0
91/85/0.95
95/86/1.0
89/79/0.98
97/56/0.96
81/-/-
0.02 substitutions per site

◀ **Fig. 1** Phylogenetic reconstruction based on partial *cox*2 sequences using minimum evolution, with support values from minimum evolution, maximum likelihood, and Bayesian inference, in the respective order. A minus sign denotes bootstrap support lower 50% or posterior probabilities lower than 0.9 for the presented or an alternate topology

Results

From 2015 to 2019, several collections of maize downy mildew were done throughout Indonesia, to clarify their phylogenetic diversity and taxonomic identity. For this, also the type specimen of *P. maydis* deposited in KRAM was requested and obtained on loan (Table 1). Phylogenetic inference (Fig. 1) revealed that the isolates were divided into two groups. The first group contained 13 isolates; 9 from Lampung (CX 1-3, KM, PRS, MK2, TGM, BJR, and BJS), 3 from Sumatera Utara (MC, TR, and DS), and 1 isolate from Jawa Barat (CK). The second group consisted of 13 isolates; 7 from Jawa Timur (Ppn, Smbh, Jnu, KD3SG, KD4SG; MDR, NGK), 2 from Jawa Tengah (KL, BY), 1 from Yogyakarta (Y), and 3 from Sulawesi Selatan (ER5, L2, and P2). These isolates were identical or less than 1% divergent from the ex-type sequence of *P. australiensis* reported by Shivas et al. (2012), but three isolates showed some divergence. Interestingly, also the type specimen of *Peronospora maydis* (Indonesia, Java, Java Tengah, Tegal, leg. Marjan Raciborski, likely 1897, KRAM O-5859(J), lectotypus hic designatus (MBT394155), as this specimen fits the site mentioned in the protologue to the description of the species (Raciborski 1897) and is the only specimen of the species preserved in KRAM). All members of the first group were revealed as the sister group to the isolates obtained from Timor-Leste that was previously thought to probably represent *P. maydis* (clade 4) (Telle et al. 2011). While this groupinng received moderate to strong support, no other.

Discussion

Identification by morphological characteristics (Shaw 1978) is still often applied for *Peronosclerospora* species in Indonesia (Hikmahwati et al. 2011; Widiantini et al. 2015; Muis et al. 2016). Based on morphological characteristics, three *Peronosclerospora* species have been reported from Indonesia, namely *P. maydis* (Bonde 1982; Rustiani et al. 2015; Muis et al. 2016), *P. sorghi* (Rustiani et al. 2015; Muis et al. 2016), and *P. philippinensis* (Bonde 1982; Rustiani et al. 2015; Muis et al. 2016). However, morphological investigations in *Peronosclerospora* are complicated by the evanescent conidiophores, which quickly vanish after the ripening of conidia. Due to this nature, conidiophores and conidia are not well-preserved in herbarium specimens, which renders comparative analyses difficult. At the same time, there is the high risk to harvest not fully mature conidiophores on which the conidia are not fully developed. In addition, conidial dimensions have been reported to be affected by environmental conditions (Dudka et al. 2007), rendering morphology-based identification difficult. Long-lasting oospores that could serve as an alternative means of identification are not produced in maize, further complicating identification of maize downy mildew.

Thus, since morphology-based identification has many constraints (Telle et al. 2011; Spencer and Dick 2002), molecular identification is crucial. In Indonesia, molecular investigation on *Peronosclerospora* has also been performed (Lukman et al. 2003; Muis et al. 2016). On the basis of simple sequence repeat (SSR) and amplified ribosomal DNA restriction analysis (ARDRA), Lukman et al. (2003) differentiated *Peronosclerospora* found in Java, Sumatra, and Sulawesi into three clusters—two from Java (cluster I and II) and one from Sumatra and Sulawesi (cluster III). Also based on SSR, Muis et al. (2016) divided *Peronoscleropora* that was obtained from Sulawesi Selatan, Sulawesi Tengah, Jawa Timur, Jawa Barat, Lampung, Sumatera Utara, Aceh, and Kalimantan Barat into 5 clusters. In neither case a morphological determination was carried out and no sequencing was done, so it remains unclear, which species the clusters could be attributed to.

Cytochrome C oxidase subunit II (*cox*2) has been reported as well-suited tool to differentiate species of oomycetes including *Peronosclerospora* (Telle et al. 2011; Thines et al. 2015; Choi et al. 2015). Telle et al. (2011) reported that specimens of *Peronosclerospora* from maize and sorghum formed four distinct phylogenetic groups different from the known *Peronosclerospora* species included in the phylogeny. Those isolates were placed in clades 4, 5, 6, and 8. In 2012, Shivas et al. (2012) introduced *P. sargae* as the name of the isolates placed in clade 5 and *P. australiensis* as the name of the isolates within clade 8. Meanwhile, the members of clade 4 and 6 were still undetermined. The members of clade 4 have been reported to infect maize in Timor-Leste and were tentatively assigned to *P. maydis* (Telle et al. 2011).

Except for one, all sequences obtained from infected maize from Sumatra investigated in this study grouped together with the ex-type sequence of *P. australiensis* with strong to maximum support, up to 100% identical to some Australian specimens. Interestingly, the type of *P. australiensis* is identical in *cox2* sequence to the type of *P. maydis*, rendering the former a synonym of the latter. However, it is also conceivable that the species is indigenous, with some species of *Sorghum*, such as Sorg*hum timorense*, from which it is also known in Australia, as natural host. Whether *P. australiensis* occurs in Indonesia naturally or has been imported from Australia, probably by infested seeds, needs to be clarified in future studies.

Using partial *cox*2 sequences, several Indonesian isolates of *Peronosclerospora* from Jawa Timur (KD3SG, KD4SG, Ppr, Smbh, Jnu), Jawa tengah (BY), Yogyakarta (Y, KL), and Sulawesi (ER5, L2, P2) were grouped with *Peronosclerospora* sp. (Telle et al. 2011) that was speculated to probably represent

P. maydis, an assumption refuted in the present study. However, as the sequences reported in Telle et al. (2011) showed only around 98% homology to this group and were consistently forming a group of its own. This suggests that the specimens found on Timor-Leste might represent an undescribed species, closely related to the unidentified *Peronosclerospora species*-from Java. *Peronoscleropsora maydis* was the first *Peronosclerospora* species reported from Indonesia (Raciborski 1897), infecting maize in the island of Java (Semangun 2008). *Peronosclerospora maydis* contr has been reported from Australia (Morschel 1980; Ramsey and Jones 1988) and their attribution to *P. australiensis* (Shivas et al. 2012) cannot be upheld anymore, suggesting that care should be taken not to spread the disease from Australia to other parts of the world. Future studies including the type specimens of other *Peronosclerospora* species will be needed to clarify the identity of other species occuring in Indonesia.

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