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Molecular phylogenetic analysis of *Peronosclerospora* (Oomycetes) reveals cryptic species and genetically distinct species parasitic to maize

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Abstract Downy mildews are amongst the most widespread and economically important pathogens of cultivated grasses in the tropics and subtropics. Despite their importance, molecular methods, particularly DNA sequence analysis, have rarely been applied to either species identification or to the determination of phylogenetic relationships between species. Here we report the presence of several cryptic species in the genus *Peronosclerospora*. Further we confirm that maize can be parasitised by several species of *Peronosclerospora*, including *P. eriochloae*, which has not been reported previously as a pathogen of maize. The presence of 14 distinct phylogenetic lineages, including three that are parasitic to maize, highlights the current fragmentary knowledge on the diversity and classification of species within *Peronosclerospora*. Species identification in *Peronosclerospora* has been traditionally based on the host genus and a set of variable morphological characteristics, which has meant that the identification of species is often unreliable. This situation is primed for the application of molecular techniques for the identification of species. One of the lineages parasitic to maize in Australia has not yet been formally described and its distribution is not known. Future investigation including a broad sampling of downy mildews from maize and other cultivated and native grasses on a world-wide basis is a prerequisite to a re-evaluation of quarantine regulations aimed at restricting or limiting their spread.

Keywords cox2 · Graminicolous downy mildews · Host range · Large nuclear ribosomal subunit (nrLSU) · Peronosporaceae · Quarantine · Sclerosporaceae

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Introduction

The graminicolous downy mildews, particularly *Peronosclerospora* spp., are a diverse group of mostly tropical oomycetes that are associated with some of the most devastating diseases of cultivated grasses, including sugarcane, sorghum and maize. These obligate pathogens pose a substantial threat to farmers in developing countries, despite some progress in resistance breeding (Kenneth 1981; Jeger et al. 1998; Kim et al. 2007). Some species, including *P. philippinensis, P. sacchari*, *P. spontanea, P. sorghi* and *P. maydis*, are listed by different plant health organisations (Cline and Farr 2006; Rossman et al. 2006; Anonymous 2008, 2009a, b) as high risk plant pathogens that should be carefully monitored and quarantined. In addition, some graminicolous downy mildews on crops have revealed a high degree of adaptability, including the acquisition of fungicide resistance (Isakeit and Jaster 2005).

Downy mildews now classified as *Peronosclerospora* have been known from maize since 1897 (Raciborski 1897). In total, 11 species are currently validly described within *Peronosclerospora* (Shaw 1978, 1980; Siradhana et al. 1980; Ryley and Langdon 2001), all of which are naturally restricted to hosts in the subfamily Panicoideae, although there are some reports of artificial transfer to Poideae (Kenneth 1981). Within the Panicoideae most species of *Peronosclerospora* are pathogens of hosts in the Andropogoneae tribe, which includes sugarcane, sorghum and maize. Only a few grass species in other tribes, for example *Pennisetum* sp. and *Panicum trypheron*, both in the Paniceae, have been reported as hosts of *Peronosclerospora*.

For most downy mildews on dicotyledons, with the exception of *Pseudoperonospora cubensis* (Choi et al. 2005; Runge and Thines 2011) a rather narrow host range has been confirmed revealing the presence of several distinct species on a specific host family including Brassicaceae (Göker et al. 2004, 2009), Fabaceae (García-Blázquez et al. 2008), and Lamiaceae (Choi et al. 2009; Thines et al. 2009). For several downy mildews on the monocotyledonous grasses, broad host ranges encompassing several genera have been confirmed in *Sclerophthora* (Telle and Thines 2011) and have also been reported for *Peronosclerospora* (Kenneth 1981). For example, *P. sacchari* has been reported to infect 9 host species, while *P. philippinensis* and *P. sorghi* have been reported from 6 and 5 host species, respectively. Conversely some grasses appear susceptible to several species of downy mildew. For example, maize (*Zea mays*) has been reported as a host for 7 species of *Peronosclerospora*, of which 5 species occurred on sorghum (*Sorghum* spp.) and 4 species on sugarcane (*Saccharum officinarum*), as stated in Kenneth (1981). In addition, pathotypes of different virulence potential seem to be present, e.g. in *P. sorghi* (Craig and Frederiksen 1980), for which it has been reported that some African varieties of sorghum are resistant to strains of *Peronosclerospora* present in North America (Prom et al. 2010).

Given the potential risk of the movement of new pathotypes and species of *Peronosclerospora*, it is crucial that their potential host ranges be determined and that species delimitation is clarified. In particular, the long-standing assumption that *P. sorghi* is parasitic to both maize and sorghum under natural conditions needs careful scrutiny. Studies using ITS fragment length differences (Yao et al. 1992), AFLPs (Perumal et al. 2006) or microsatellites (Perumal et al. 2008) have revealed a significant diversity among *Peronosclerospora* isolates, and some clustering was observed, giving a first hint to potential species boundaries in *Peronosclerospora*.

Species delimitation in *Peronosclerospora* is notoriously difficult due to the fact that sporangia and sporangiophores are evanescent, leaving oospores and haustoria as the only taxonomically informative structures in herbarium specimens (Kenneth 1981; Thines 2006). Sexual reproduction has not been observed for several species, so morphological characteristics of gametangia and oospores are not available. Naming of isolates in these cases has been based on host and geographic origin. Thus, their taxonomy has yet to be substantiated. For example, *P. sorghi*, *P. maydis*, *P. saccharum*, and *P. philippinensis* have all been reported to attack sorghum, maize, and sugarcane (Kenneth 1981). Species with an unknown sexual stage include *P. philippinensis* and *P. spontanea*, both of which have been reported as potential pathogens of maize. Because of the paucity of morphological characters for species delimitation, it is currently impossible to provide an unequivocal key for species delimitation in *Peronosclerospora*. In addition, it should be noted that sporangial dimensions, which have been used as a main characteristic for species delimitation in *Peronosclerospora*, especially for those species without a known sexual stage (Weston

1921), seem variable for some species and may be influenced by climatic conditions (Kimigafukuro 1979; Leu 1973), host species and variety (Bock et al. 2000).

The phylogenetic relationships of the genera of downy mildews on grasses are so far unresolved (Thines et al. 2008), but all genera hitherto described appear distinct (Riethmüller et al. 2002; Göker et al. 2003; Hudspeth et al. 2003; Thines et al. 2006, 2007, 2008). Molecular phylogenetic investigations offer additional characteristics suitable for identification of species of *Peronosclerospora* that may unequivocally resolve their diversity and natural host range. The aim of this study was to clarify whether species of *Peronosclerospora* are phylogenetically distinct and if molecular evidence existed that could confirm the presence of cryptic species on a specific host as well as the presence of specific species on more than one host genus.

Materials and methods

The specimens used in this study are listed in Table 1. Sample preparation, DNA extraction and PCR reaction were carried out according to Telle and Thines (2008), with some exceptions. The lysis buffer of the Analytikjena plant DNA extraction kit (innuPREP Plant DNA Kit, Analytikjena, Germany) was modified with 10 mM PTB and Mango Taq DNA Polymerase (1U/25 µl; Bioline, Germany) was used for amplification. Forward and reverse primers for amplification of cox2 were those described by Hudspeth et al. (2000) and primers used for the amplification of partial nrLSU were LR-0-R and LR6-O described by Moncalvo et al. (1995) and Riethmüller et al. (2002), respectively. PCR products were separated on agarose gels and purified with a commercial purification kit (illustra GFX PCR DNA and Gel Band Purification kit; GE Healthcare, USA). Sequencing was carried out by a commercial sequencing provider (GATC Biotech, Germany).

Sequences were aligned for each region individually using mafft (v6.811b, Katoh and Toh 2008a), using the Q-INS-i option (Katoh and Toh 2008b). For phylogenetic analysis, leading and trailing gaps caused by missing data were removed and the sequences concatenated. MEGA 4.0 (Tamura et al. 2007) was used for Minimum Evolution (ME) inference, with default settings except for assuming pairwise deletion, and using the Tamura-Nei nucleotide substitution model. For inferring tree robustness, 1000 bootstrap replicates were carried out. For Maximum Likelihood (ML) phylogenetic inference, RAxML (Stamatakis 2006) was used as implemented on the RAxML webservers (Stamatakis et al. 2008). The web-server at http://phylobench.vital-it.ch/raxml-bb/index.php was used with the gamma model of rate heterogeneity in effect and maximum likelihood search for the best-scoring tree. The bootstraped trees of five runs were combined and a consensus tree was computed using MEGA for assessing bootstrap support. Bayesian analysis was performed using MrBayes (Parallel MrBayes @ BioHPC ver 3.1.2, http://cbsuapps.tc.cornell.edu/mrbayes.aspx) with MrBayes block generated by GUMP at Auburn University (http://gump.auburn.edu/srsantos/mrbayes_form/) with the following settings deviating from the standard settings—nst=6, rates=gamma. The Markov chain Monte Carlo search was run with 4 incrementally heated chains for 10,000,000 generations, with trees sampled every 1000th generation. The first 2500 trees sampled this way were discarded, to ensure scoring trees from the stationary phase. The remainder of the trees were used to calculate a majority rule consensus tree and for assessing posterior probabilities of the nodes. A clade was defined as a sequence or group of sequences clustering together with high support in at least one of the analyses, with no highly supported more basal nodes within *Peronosclerospora*.

Results

In the molecular phylogenetic reconstruction based on partial *cox2*, and nrLSU sequences (Fig. 1), 12 phylogenetically distinct lineages, which can be placed in 8 monophyletic clades, were revealed. Clade 1 is comprised of a basal lineage parasitic to *Panicum laevinode* and three closely related phylogenetic lineages parasitic to *Eriochloa pseudoacrotricha* and maize. *Peronosclerospora eriochloae* on *Eriochloa* is almost identical in sequence (99.79%, 1423 of 1426 nucleotides) to one of the specimens of this clade collected from maize (BRIP 22977). A specimen of *P. sorghi* from India constitutes clade 2, with unresolved phylogenetic affinity to the other species of *Peronosclerospora*. Clade 3 contains the specimens of *P. noblei* from *Sorghum leiocladum*. At

Table 1 Specimen used in this study

Species	Host	Collection details			GenBank accession no.	
		Voucher or identity	Locality	Date	nrLSU	*cox*2
Peronosclerospora sp.	*Sorghum plumosum*	BRIP 46738	Australia, WA	2005, May	HQ261768	HQ261795
Peronosclerospora sp.	*Sorghum plumosum*	BRIP 46736	Australia, WA	2005, May	HQ261770	HQ261797
Peronosclerospora sp.	*Sorghum timorense*	BRIP 49819	Australia, WA	2007, Apr	HQ261779	HQ261806
Peronosclerospora sp.	*Sorghum timorense*	BRIP 49816	Australia, WA	2007, Apr	HQ261780	HQ261807
Peronosclerospora sp.	*Zea mays*	BRIP 46815	Australia, NT	2005, Aug	HQ261766	HQ261793
Peronosclerospora sp.	*Zea mays*	BRIP 46527	Australia, NT	2005, May	HQ261767	HQ261794
Peronosclerospora sp.	*Sorghum plumosum*	BRIP 46735	Australia, NT	2005, May	HQ261769	HQ261796
Peronosclerospora sp.	*Sorghum plumosum*	BRIP 46698	Australia, NT	2005, May	HQ261771	EU116049[*2]
Peronosclerospora sacchari	*Saccharum* sp.	BRIP 44241 a	East Timor	2004, Apr	HQ261764	EU116052[*2]
Peronosclerospora miscanthi	*Miscanthus japonicus*	NY[*1]	Philippines, Luzon	1930, Apr	HQ261784	HQ261811
Peronosclerospora sp.	undetermined	BRIP 49806	Australia, NT	2006, Jun	HQ261772	HQ261799
Peronosclerospora sp.	*Sorghum timorense*	BRIP 27691	Australia, NT	2000, Mar	HQ261782	HQ261809
Peronosclerospora sp.	*Zea mays*	BRIP 44938 b	East Timor	2004, Feb	HQ261776	HQ261803
Peronosclerospora sp.	*Zea mays*	BRIP 44939 a	East Timor	2004, Feb	HQ261777	HQ261804
Peronosclerospora sp.	*Zea mays*	BRIP 44937 a	East Timor	2004, Feb	HQ261775	HQ261802
Peronosclerospora sp.	*Zea mays*	BRIP 44924	East Timor	2004, Feb	HQ261774	HQ261801
Peronosclerospora sp.	*Zea mays*	BRIP 44922	East Timor	2004, Feb	HQ261773	HQ261800
Peronosclerospora sp.	*Zea mays*	BRIP 44941 b	East Timor	2004, Feb	HQ261778	HQ261805
Peronosclerospora noblei	*Sorghum leiocladum*	HOH HUH 891	Australia, QLD	2007, Jan	HQ261785	EU116058[*2]
Peronosclerospora noblei	*Sorghum leiocladum*	BRIP 17464	Australia, QLD	1991, Mar	HQ261783	HQ261810
Peronosclerospora sorghi	*Sorghum bicolor*	HOH HUH 897	India, Karnataka	2005	HQ261763	EU116055[*2]
Peronosclerospora eriochloae	*Eriochloa pseudoacrotricha*	FR-0046005	Australia, QLD	2008	HQ261786	HQ261813
Peronosclerospora cf. *eriochloae*	*Zea mays*	BRIP 22977	Australia, QLD	1995, Nov	HQ261765	HQ261792
Peronosclerospora cf. *eriochloae*	*Zea mays*	BRIP 22711	Australia, QLD	1995, Apr	HQ261781	HQ261808
Peronosclerospora sp.	*Panicum laevinode*	DAR 35733	Australia, NSW	1980, Mar	HQ261787	HQ261814
Sclerospora graminicola	*Pennisetum glaucum*	FR-0046006[*3]	India, Maharashtra	1997	HQ261761	HQ261788
Sclerospora graminicola	*Pennisetum glaucum*	FR-0046007[*4]	India, Gujarat	1998	HQ261762	HQ261789

[*1] Stevens' Philippine Fungi, Island of Luzon, No. 811. [*2] sequences obtained in a previous study (Thines et al. 2008). [*3] strain designation sg150. [*4] strain designation sg200

least three distinct species were observed from *Sorghum*, one parasitic to *S. leiocladum* (clade 3), another parasitic to *S. stipoideum* (clade 5) and a third (and potentially fourth) parasitic to *S. plumosum* and *S. timorense* (clade 8). Clade 4 is comprised of a group of specimens from *Zea mays* from East Timor, which show significant genetic divergence from an Indian specimen of *P. sorghi* from *S. bicolor* (clade 2), and thus is referred to as *Peronosclerospora* sp. in Fig. 1.

Clades 5 to 8 form a monophylum containing specimens that are grouped together with weak support (57/63/0.74) from several host species in the Andropogoneae. Clade 5 is represented by a single specimen from *S. stipoideum*, which is sister to clades 6 to 8 that are grouped together with moderate support (82/82/0.92). Clades 6 and 7 are grouped together with weak support (52/-/0.79). Clade 6 contains a single specimen from an undetermined host, while clade 7 is represented by *P. miscanthi* and *P. sacchari*, which are grouped together with high support (99/96/1.00) in all analyses. Clade 8 contains specimens that received moderate to high support (78/94/1.00) from *S. plumosum* and *S. timorense*, as well as from two accessions from maize. This clade is further divided into two subclades, one containing

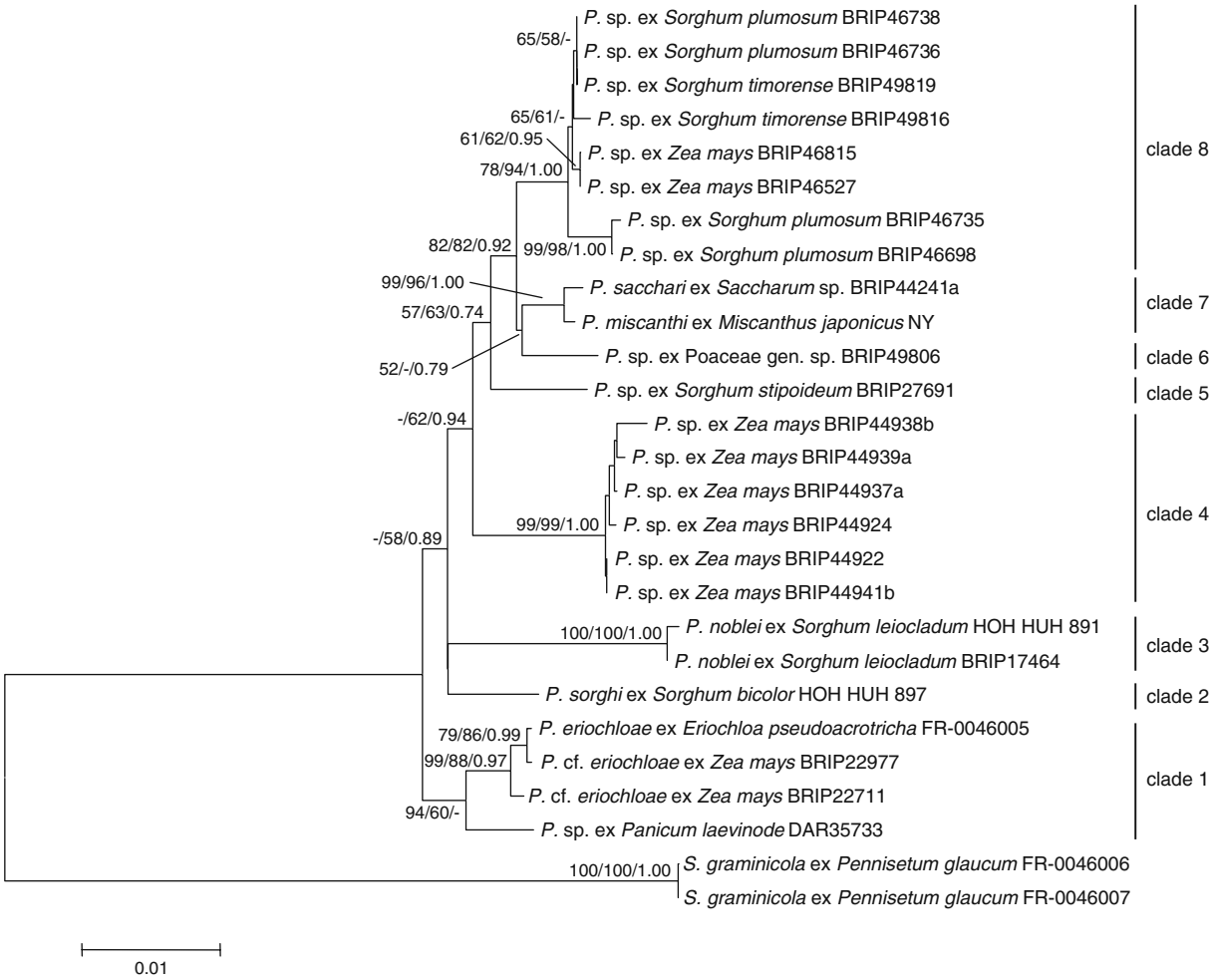

Fig. 1 Phylogenetic reconstruction of *Peronosclerospora* (ME) with combined nrLSU and *cox*2 sequences and support values in ME, ML and Bayesian Analysis given on branches in the respective order, *P.* = *Peronosclerospora*, *S.* = *Sclerospora*

solely specimens from *S. plumosum*, while the other contains specimens from *S. plumosum*, *S. timorense* and maize. The integrity of these subclades received high (99/98/1.00) and low support (65/61/-), respectively.

Discussion

This study supports previous reports about the potential of several species of *Peronosclerospora* to also infect maize (Frederiksen and Renfro 1977; Kenneth 1981), while at the same time revealing the presence of a variety of cryptic species. For example, we found at least two distinct and undescribed species of *Peronosclerospora* infected *Sorghum* species, which are native to northern Australia and restricted to Australasia (Spangler 2003). Ryley and Langdon (2001) noted that *S. leiocladum* was the only confirmed host for *P. noblei*. We found that one of the cryptic species occurring on the two native Australian grasses, *S. plumosum* and *S. timorense*, also occurred on maize. This result supports the investigations of Ramsey and Jones (1988), who used infection studies and field observations to conclude that downy mildew on *S. plumosum* might act as a reservoir for the infection of maize. We have found that these infections can occur naturally. Consequently, an eighth undescribed species of *Peronosclerospora* is reported as a pathogen of maize, in addition to *P. heteropogoni*, *P. maydis*, *P. miscanthi*, *P. philippinensis*, *P. sacchari*, *P. sorghi*, and *P. spontanea* (Kenneth 1981).

In addition to the newly discovered cryptic species, two phylogenetically distinct lineages of *Peronosclerospora* were found to infect maize. One of these contained pathogens of maize from East Timor that may represent *P. maydis*. It is noteworthy that an Indian specimen of the morphologically similar species *P. sorghi* on its type host *Sorghum bicolor*, was shown in our phylogenetic reconstruction to be distinct from the maize-infecting lineages. *Peronosclerospora maydis* has been thought to be absent or extinct from North America (Cline and Farr 2006; Rossman et al. 2006), and quarantine regulations are in effect. A broader sampling of *P. maydis* from several countries in which the disease has been reported is necessary to clarify the distribution of this pathogen.

Peronosclerospora eriochloae was described from *Eriochloa pseudoacrotricha*, which differs from all other species of *Peronosclerospora* (Ryley and Langdon 2001). It is noteworthy that this pathogen can infect maize, as revealed by the clustering of a specimen from a progeny of the type specimen with a specimen collected from maize, thus representing the ninth species occurring on this host.

The high susceptibility of maize to various downy mildew species might reflect the original absence of *Peronosclerospora* from the American continents (Spencer and Dick 2002), thus there has been no selection pressure for the evolution of downy mildew resistence in maize and its wild relatives. In addition, the long-term domestication of maize may have caused a bottleneck, eliminating some downy mildew resistance from the cultivated gene pool and in addition seed yield may be negatively correlated with resistance (Tian et al. 2003), thus raising the likelihood of inadvertedly selecting against resistance in breeding efforts. Multiple species of *Peronosclerospora* have been reported as pathogens of maize, sugarcane and other C4 grasses (Waterhouse 1964). For example it has been reported that both *P. sacchari* and *P. miscanthi* both occur on sugarcane. The two species are shown to be closely related in this study, with a sequence divergence of 0.92% (13 out of 1426). These two species also have similar oospore morphology based on their original descriptions, but differ with respect to their conidiophores (Waterhouse 1964). Further investigations are necessary to evaluate their status as distinct species.

Several species of *Peronosclerospora* (*P. philippinensis*, *P. maydis*, *P. spontanea*, and *P. dichanthiicola*) are only known from their asexual stages, which can be affected by climatic conditions (Kimigafukuro 1979; Iwata 1942; Frederiksen and Renfro 1977) and host species (Waterhouse and Brothers 1981; Runge and Thines 2011). A systematic scrutiny of the variability of conidiophores and conidia morphology in addition to a broad sampling from their respective hosts is necessary to determine whether these species represent distinct lineages or are conspecific with other species. It is highly likely that species reported or described from maize (*P. heteropogonis*, *P. maydis*, *P. miscanthi*, *P. philippinensis*, *P. sacchari*, *P. sorghi*, and *P. spontanea*), have other natural hosts, as maize is not native to the natural range of *Peronosclerospora* (Spencer and Dick 2002). The original hosts of most of these species await discovery, with the possible exception of *P. heteropogoni* which infects *Heteropogon contortus*, a grass widespread in tropical and subtropical regions of the world. The evidence of this study together with previous reports on the susceptibility of maize to several species of *Peronosclerospora*, indicates that maize is serving as a catch-all crop for indigenous downy mildews. Species of *Peronosclerospora* pose a serious threat to agriculture, and utmost care should be taken to prevent their spread from their native range to other maize growing countries. We have shown that accepted *Peronosclerospora* species may actually represent complexes of cryptic species. Unravelling these species with molecular and morphological methods is essential for the reliable diagnosis and future risk assessment of the downy mildews on grasses, particularly those on maize, sugarcane, and sorghum.

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