Abstract

Background: A recent outbreak of sorghum downy mildew in Texas has led to the discovery of both metalaxyl resistance and a new pathotype in the causal organism, *Peronosclerospora sorghi*. These observations and the difficulty in resolving among phylogenetically related downy mildew pathogens dramatically point out the need for simply scored markers in order to differentiate among isolates and species, and to study the population structure within these obligate oomycetes. Here we present the initial results from the use of a biotin capture method to discover, clone and develop PCR primers that permit the use of simple sequence repeats (microsatellites) to detect differences at the DNA level.

Results: Among the 55 primers pairs designed from clones from pathotype 3 of *P. sorghi*, 36 flanked microsatellite loci containing simple repeats, including 28 (55%) with dinucleotide repeats and 6 (11%) with trinucleotide repeats. A total of 22 microsatellites with CA/AC or GT/TG repeats were the most abundant (40%) and GA/AG or CT/TC types contribute 15% in our collection. When used to amplify DNA from 19 isolates from *P. sorghi*, as well as from 5 related species that cause downy mildew on other hosts, the number of different bands detected for each SSR primer pair using a LI-COR DNA Analyzer ranged from two to eight. Successful cross-amplification for 12 primer pairs studied in detail using DNA from downy mildews that attack maize (*P. maydis & P. philippinensis*), sugar cane (*P. sacchari*), pearl millet (*Sclerospora graminicola*) and rose (*Peronospora sparsa*) indicate that the flanking regions are conserved in all these species. A total of 15 SSR amplicons unique to *P. philippinensis* (one of the potential threats to US maize production) were detected, and these have potential for development of diagnostic tests. A total of 260 alleles were obtained using 54 microsatellites primer combinations, with an average of 4.8 polymorphic markers per SSR across 34 *Peronosclerospora*, *Peronospora* and *Sclerospora* spp isolates studied. Cluster
isolates collected from previously resistant hosts revealed acetyl-(DL-alaninate] fungicide, which had been used as metalaxyl [methyl N- (2, 6-dimethylphenyl)-N-(methoxy-
isolates from the outbreak were found to be resistant to during the spring of 2001 and again in 2002 [6].

The population structure of P. sorghi has been previously addressed with physical characters, such as spore size or pathotype, which require comparative disease responses on a series of host differential cultivars [11,12]. Conventional methodologies for the detection of different pathotypes of downy mildews of cereals have not been satisfactory particularly for the identification of organisms at extremely low propagule numbers and are cumbersome for use with obligate parasites (such as plant-pathogenic rusts and mildews), which cannot be cultured. Hence, in tropical climates, symptoms of Peronosclerospora can be confused with those from related downy mildew pathogens, including P. maydis, P. sacchari and P. philippinensis. Yao et al. [13] showed that clones of P. sorghi DNA cross-hybridized well on Southern blots with DNA from the other Peronosclerospora species. While few differences were detected among the RFLP patterns within P sorghi, different patterns were seen for the different species. Similarly, ITS-2, an internal transcribed spacer of ribosomal genes
was different in size [14]. While potentially useful in species identification, more easily scored and highly variable markers from locations throughout the genome are needed for differentiating among closely related pathotypes, and to tag genes that contribute to host specificity, race specificity, and virulence. Although polymorphisms were detected in SDM pathotypes using RAPD-PCR, many of the short (10 mer) primers resulted in monomorphic banding patterns. A larger problem involved inconsistently amplified bands that varied greatly in intensity, even in duplicate experiments [15]. As a consequence, a more consistent procedure was sought in the present investigation for the development of microsatellite markers.

Simple sequence repeat (SSR), or microsatellites, are hypervariable and dispersed in the form of long arrays of short tandem repeat units throughout the genome [16,17]. These SSRs provide codominant Mendelian markers, much more powerful than dominant markers and can be used to determine population genetic structure, kinship, reproductive mode, and genetic isolation [18,19]. When compared with several other marker types, SSR markers were superior for genetic characterization in species identification, more easily scored and highly variable markers from locations throughout the genome are needed for differentiating among closely related pathotypes, and to tag genes that contribute to host specificity, race specificity, and virulence. Although polymorphisms were detected in SDM pathotypes using RAPD-PCR, many of the short (10 mer) primers resulted in monomorphic banding patterns. A larger problem involved inconsistently amplified bands that varied greatly in intensity, even in duplicate experiments [15]. As a consequence, a more consistent procedure was sought in the present investigation for the development of microsatellite markers.

Methods
Pathogen samples and DNA isolation
A total of 19 isolates of *Peronosclerospora sorghi* including P1 (metalaxyl susceptible), P3 and P6 (both metalaxyl resistant and susceptible) pathotypes collected in different years from different parts of Texas where the disease outbreak occurred were included in this study. In addition, DNA samples from *P. philippinensis* (one), *P. maydis* (five), *P. sacchari* (three) were obtained from Dr. Douglas G. Luster, USDA/ARS, Foreign Disease-Weed Science Research Unit, Ft. Detrick, Maryland, USA and DNA samples of *Sclerospora graminicola* (five) and *Peronospora sparsa* (one) available in Dr. Magill’s lab, Dept. of Plant Pathology & Microbiology, Texas A&M University, College Station, Texas, USA were also included in the present study (Table 1). For DNA isolation, conidia were collected from infected leaves as follows: leaves were washed with cold sterile water and placed abaxial side down on a screen in a petri plate, covered with moist paper towels and allowed to sporulate in the dark overnight at 23°C [26]. Spores collected in sterile water were used for DNA extraction. After removing excess water, conidial suspensions were washed a few times with 70% ethanol and frozen for lyophilization. The dried sample was powdered in liquid nitrogen using a mortar and pestle. DNA was extracted using a MasterPure Yeast DNA Purification kit (EPICENTRE Biotechnologies, Madison, WI) and the DNA was diluted to working concentration of 2.5 ng/μL by adding 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microsatellite isolation
A microsatellite-enriched library was prepared by a simplified protocol based on the biotinylated-oligonucleotide capture methods of [27] and [28]. Further, several steps in the biotin capture protocol were modified as detailed by Reddy et al. [29] to optimize the frequency of microsatellite repeats among captured genomic DNA fragments. In our protocol, no size fractionation steps or radiolative hybridizations were employed. A 500 ng sample of genomic DNA from SDM pathotype 3 was digested for 3 h in a single reaction mixture containing restriction endonucleases *HaeIII*, *RsaI*, and *DraI* (20 units of each), as well as 50 ng of RNaseA. This digestion resulted in a diverse population of blunt-ended restriction fragments with an average size of ~550 bp. Digested DNAs were purified using a QIA-quick PCR purification column (Qiagen, Valencia, CA), eluted with 50 μL of 5 mM Tris-pH 8.0, then dried completely under vacuum. The double-stranded adaptor molecule AP11/12 was prepared by mixing equal molar amounts of oligonucleotides AP11 (5’TCCTGCTTAGATCCGACA3’) and AP12 (5’pTAGTCCAGATCTAAGCA-3’, where p = 5’ phosphate), heating to 94°C, then cooling to 25°C over a period of 5 h. Digested genomic DNA fragments were resuspended in a 30-μL ligation reaction containing 100 ng of AP11/12 double-stranded adaptor and 30 Weiss units of T4 DNA ligase. Ligation was carried out at 14°C for 16 h. Preamplification of adaptor-ligated products was performed using 2 μL of the ligation reaction as a template for 10 cycles of PCR in a 50-μL reaction volume using the
### Table 1: Downy mildew isolates of *Peronosclerospora*, *Sclerospora* and *Peronospora* spp analyzed in this study

| Iso. No | Pathotype/DNA | Metalaxyl reaction | Host                  | Location & year       |
|---------|---------------|---------------------|-----------------------|-----------------------|
| 1       | P1            | Sensitive           | Tx7978-sorghum         | Green house, TAMU, TX |
| 2       | P3            | Sensitive           | Tx430-sorghum          | Green house, TAMU, TX |
| 3       | P3            | Sensitive           | Whart-A-sorghum        | Wharton County, TX – 2001 |
| 4       | P3            | resistant           | CR 360A – sorghum      | Wharton County, TX – 2002 |
| 5       | P3            | resistant           | CR 459A – sorghum      | Wharton County, TX – 2002 |
| 6       | P3            | resistant           | Fucik B – sorghum      | Wharton County, TX – 2002 |
| 7       | P3            | resistant           | Merta A – sorghum      | Wharton County, TX – 2002 |
| 8       | P3            | resistant           | Wesla A – sorghum      | Weslaco, TX – 2002    |
| 9       | P3 or P6      | resistant           | Grain sorghum          | Wharton County, TX – 2004 |
| 10      | P6            | resistant           | Johnson grass          | Jackson county TX – 2007 |
| 12      | P6            | resistant           | Grain sorghum          | Wharton County, TX – 2007 |
| 13      | P6            | resistant           | Grain sorghum          | Wharton County, TX – 2006 |
| 14      | P3            | resistant           | Grain sorghum          | Wharton County, TX – 2006 |
| 15      | P3 or P6      | resistant           | Grain sorghum          | Wharton County, TX – 2006 |
| 16      | P1            | Unknown             | Johnson grass          | Nueces county, TX – 2007 |
| 17      | P6            | resistant           | Johnson grass          | Wharton county, TX – 2007 |
| 18      | P6            | resistant           | Grain sorghum          | Wharton county, TX – 2006 |
| 19      | P3 or P6      | resistant           | Grain sorghum          | Jackson county, TX – 2006 |
| 21      | P1            | Sensitive           | Grain sorghum          | Nueces county, TX – 2007 |

**Peronosclerospora maydis – Maize downy mildew isolates**

| DNA #   | -                           | Corn                  | Suwan Farm, Thailand-1975 |
|---------|------------------------------|-----------------------|----------------------------|
| 22      | DNA # 5                      | -                     |                            |
| 23      | DNA # 6                      | -                     | Malang, Indonesia – 1987  |
| 24      | DNA # 7                      | -                     | West Java, Indonesia – 1987 |
| 25      | DNA # 8                      | -                     | Suwan Farm, Thailand-1985 |
| 26      | DNA # 9                      | -                     | Suwan Farm, Thailand-1985 |

**Peronosclerospora philippinensis isolate**

| DNA #   | -                           | Corn                  | Los Banos, Philippines, 1979 |
|---------|------------------------------|-----------------------|-------------------------------|
| 27      | DNA # 1                      | -                     |                               |
single primer AP11. An annealing temperature of 55°C was employed in all PCR reactions.

Approximately 100 ng of the preamplified product was then added to a single reaction mixture containing 6× SSC (0.9 M NaCl, 90 mM sodium citrate, pH 7), 0.1% SDS (sodium dodecyl sulfate), and 200 ng each of biotinylated oligos b(TA)30, b(CA)20, b(GA)20, and b(AGA)15, b(TGA)15, b(ACA)15, (b = 5’ biotinylation). After denaturation at 95°C for 5 min, preamplified genomic DNA fragments were annealed in the presence of biotinylated oligonucleotides for 1 h at 60°C, then added to 200 μg of fresh streptavidin-coated paramagnetic beads (Promega, Madison, WI) previously equilibrated with 6× SSC. Beads were incubated at 60°C with gentle agitation for 15 min, then the liquid was removed by separation using a magnetic stand (Stratagene, San Diego, CA). Beads were washed twice in 300 μL of 6× SSC, 0.1% SDS for 15 min at room temperature with gentle agitation. Beads were further washed twice in 300 μL of 6× SSC, 0.1% SDS for 15 min at 60°C with gentle agitation. Finally, beads were briefly washed twice with 6× SSC at room temperature. After removing the final wash, captured DNAs were eluted from the beads with the addition of 100 μL of 60°C 0.1 M NaOH. After neutralization with 100 μL of 1 M Tris-pH 7.5, captured DNAs were desalted and equilibrated with 10 mM Tris-pH 8.0, 1 mM EDTA-pH 8.0 (to a final volume of ~50 μL) using a 100-kDa MW cutoff size filtration column (Millipore, Bedford, MA). Five μL of desalted DNA sample were used as a template for 30 cycles of PCR in a 50-μL reaction volume using primer AP11. Six microliters of the resulting PCR reaction (~60 ng) was cloned into the TA-cloning vector pCR4-TOPO through topoisomerase mediated ligation (Invitrogen, San Diego, CA) and transformed into chemically competent Escherichia coli TOPO10. Recombinant colonies were identified by positive selection through insertional inactivation of the ccdB (control of cell death) open reading frame. Colonies were transferred to 96-well microtiter plates for archival storage.

**Sequence Analysis**

Recombinant bacterial colonies were inoculated into 300 μL of LB amp media in a 96-well 0.6-mL-deep plate (Marsh Bioproducts, Rochester, NY). Cultures were agitated at 500 rpm in a HiGro high-density shaking-incubator (GeneMachines, San Carlos, CA) for 16 to 18 h at 37°C. DNA sequencing was performed by RCA (rolling circle amplification) using TempliPhi 100 Amplification Kit (GE Healthcare Bio-Sciences Co. Piscataway, NJ). 0.5 μL of the bacteria was added into 5 μL of sample buffer. The sample was denatured at 95°C for 3 minutes, and then cooled to room temperature or 4°C. The denatured sample was combined with 5 μL of reaction buffer and 0.2
μl enzyme mix. DNA was incubated at 30°C for 18 hrs for Templifli reaction. The amplified DNA was diluted 50 μl of water. 2 μl from the diluted RCA product was sequenced using BigDye terminator cycle sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA) using 10 pmol of M13-forward and M13-reverse primer in a 10-μl reaction. Standard cycle sequencing conditions were employed. Sequencing products were purified by DyeEX 96 kit (Qiagen, Valencia, CA). Electrophoretic separation of sequencing products was performed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Assembly of double-stranded DNA sequence contigs from each clone, and identification of redundancy and overlaps between clones was performed using Sequencher 3.0 (Gene Codes, Ann Arbor, MI). Microsatellite analysis for the identification of sequences with perfect and imperfect repeats and simple and compound repeat motifs were identified using Simple Sequence Repeat Identification Tool (SSRIT) database [30].

**Sequence homology search**

The sequences of SDM microsatellites were subjected to homology searches using BLASTN against nucleotide collection (nt/ntr) and Expressed Sequence Tags (est_others) at National Center for Biotechnology Information (NCBI; [31]).

Under Algorithm parameters the following options were chosen to improve the results.

**General parameters**

Max target sequences – 100; Short queries – automatically adjust parameters for short input sequences; Expected threshold – 10 & Word size – 11

**Scoring parameters**

Match/Mismatch scores – 2,-3; Gap costs – Existence: 5, Extension: 2

**Filters and Masking**

Filter – low complexity regions; species-specific repeats for fungi

Mask – Mask for lookup table only options

**Design of primers and PCR amplification**

Primers were designed to the flanking region of the SSR using PRIMER 3 software[32]. Primers were designed without repetitive DNA and with a base composition of greater than 40% G+C with annealing temperature between 52°C and 58°C to yield amplification product between 100 and 250 bp. Oligonucleotide primers were synthesized by Integrated DNA Technologies, Coralville, IA, USA. Primers selected by these criteria were evaluated further for melting temperature, internal structure, and propensity for primer-dimer formation using publicly accessible Worldwide Web resources (Sigma-Genosys, The Woodlands, TX). PCR amplification was carried out in 10 μL containing 5.5 ng of genomic DNA, 1 μL 10× PCR buffer (Promega), 0.8 μL of 2.5 mM dNTP mix, 1 μL each of 1 pM/μL forward and reverse primers, 1 μL of 25 mM MgCl₂, and 1 U of Taq polymerase. PCR was performed for an initial denaturation of 2 min at 95°C followed by 35 cycles of 45 s at 95°C, 45 s at the appropriate annealing temperature for each microsatellite primer pair as highlighted in Table 1 and 60 s at 72°C, and a final extension of 20 min. at 72°C.

**Gel Analysis**

The developed SSR primers with 4% SFR (super fine resolution) agarose (AMRESCO, Solon, Ohio, USA) gel electrophoresis system running with 1× TBE buffer in 4°C using circulatory cool buffer system was used for better resolution of polymorphic differences between downy mildew isolates. The SSR amplification products were also analyzed using a LI-COR-NEN® Model 4300 -dual-dye automated DNA Analyzer. Following amplification, an equal volume (5 μl) of PCR products of two sets of SSRs one labeled using the IRD-700 nm and another with IRD-800 nm fluorogenic forward primer were mixed. Basic fusion dye (2 μl) (LI-COR) was added to each pooled sample and the samples were denatured for 5 min at 95°C. Each sample (1 μl) was loaded on a 6.5% polyacrylamide gel containing 7 M urea. Gels were cast using LI-COR 25-cm plates with 0.25-mm-thick spacers and comb. Electrophoresis was performed at a constant power of 40 W and a constant temperature of 47.5°C for 2 h.

**Statistical analysis**

Microsatellite fragments were scored as present (= 1) or absent (= 0) across the 34 Peronosclerospora, Peronospora and Sclerospora spp. isolates. The resulting binary matrix was used to construct a similarity matrix using the Jaccard coefficient, GS (ij) = a/(a + b + c) (17), where a is the number of fragments shared by i and j, b is the number of fragments present in i and absent in j, and c is the number of bands present in j and absent in i. Cluster analysis was performed using the unweighted pair group with arithmetic means method (UPGMA) [33]. PAUP 4.0* was used to generate 1000 bootstrap replicates for testing the reliability of the dataset and to draw a consensus tree [34]. Ordination analysis was performed to study the relatedness within a matrix by converting the pairwise distance into Eigen vectors and values. Cluster analyses, ordination analyses and the Mantel test were performed using NTSYSpc (NTSYS – for Numerical Taxonomy SYStems) version 2.1 [35].

**Results**

A collection of 513 colonies, picked at random from primary transformation plates, was inoculated into 96-well culture plates for high throughput sequencing. Of the 513
clones sequenced, 170 (33%) were redundant with other clones in the same collection. A total of 73 out of 343 clones (21%) did not contain repeat motifs. Of the remaining 270 nonredundant clones, Sequence analysis showed that virtually all of the inserts contained microsatellite repeat motifs matching one or more of the biotinylated oligonucleotides used in the selection process. Out of 270 clones, 144 insert fragments (53%) were truncated within, or at the end of, a microsatellite repeat. Of the remaining 126 non redundant clones with unique sequences flanking both ends of the microsatellite repeats, 55 were selected for further analysis on the basis of the following criteria: (i) the length of the microsatellite was five or greater in dinucleotide and four in trinucleotide repeat units and; (ii) the unique 5’ and 3’ flanking sequences were both of suitable structure and composition for the design of efficient primers. Microsatellites with flanking sequences that were highly repetitive in nature and/or had high A+T rich nucleotides were eliminated.

Thirty six microsatellite loci containing a simple repeat type made up 66% of the DM collection, including 28 (55%) with dinucleotide repeat types and 6 (11%) with trinucleotide repeat types (three CAA and one each of GTT, AGG and ACA). Of the microsatellites containing a simple repeat type, 94% had "perfect" repeats (except DM9 and DM16), uninterrupted by nonrepeat nucleotides. The remaining 19 SSR loci (39%) of the collection were composed of "compound repeats" consisting of more than one repeat type at a single locus and all were interrupted by nonrepeat nucleotides and hence considered as "imperfect" repeats (Table 2). All the compound repeats were combinations of dinucleotide repeat motifs except DM45 (trinucleotide) and DM49 (combination of tetra and dinucleotide) repeat motifs. A total of 22 microsatellites with the CA/AC or GT/TG repeat type were the most abundant (40%) and the GA/AG or CT/TC type contributes 15% in our collection.

Of the 55 cloned SSR sequences queried in BLASTN searches of the NCBI non-redundant genomic and EST databases (as of July 4, 2008), only four clones DM21, DM28, DM53 and DM55 were found to show similarity to previous entries and these were to sequences of Phytophthora and Pythium species which are also plant pathogenic oomycetes. Details are presented in Table 3. These results indicate that two of the cloned sequences (DM53 and DM54) isolated from P. sorghi contain incomplete portions of genes found in known retrotransposon elements.

Primers were designed for the 55 selected microsatellite loci using the criteria described in materials and methods. These primers (for Table 4 see additional file 1) had an average length of 20.7 nucleotides, with an average G+C content of 49% and annealing temperature in a range of 50–55°C. Predicted cloned products ranged in size from 106 (DM52) to 249 (DM21) bp. These 55 new microsatellite loci described in Table 4 (see additional file 1) were designated as "DM" (Downy Mildew). One of the 55, DM15 was not amplified in any of the pathotypes when resolved either in SFR agarose or LI-COR polyacrylamide gels. DM17, 27, 40, 41, 45 and 48 were resolved with faint expression in SFR agarose gels and with fine resolution in LI-COR polyacrylamide gels. When used with 34 Peronosclerospora and Sclerospora spp isolates, a total of 260 alleles were obtained using 54 microsatellites primer combinations in LI-COR polyacrylamide gels, with an average of 4.8 polymorphic markers per SSR. In comparison, only 128 alleles were visible using 48 SSRs in SFR agarose gels with an average of 2.7 polymorphic markers per SSR across the same set of isolates. Four SSRs (DM12, 19, 30 and DM38) showed monomorphic expression in SFR agarose gels but polymorphisms were detected in LI-COR poly-acrylamide gels. SFR agarose gel data for 54 SSRs across species were not used further in this study.

Of the 54 SSR primer pairs, 50 produced amplicons in Peronosclerospora sorghi, 41 in P. maydis, 29 in P. sacchari, 33 in P. philippinensis, 30 in Peronospora sparsa and 37 in Sclerospora graminicola downy mildew isolates (Table 5). Twelve SSR primer pairs (DM 9, 12, 13, 14, 16, 19, 31, 35, 49, 53 and DM54) amplified products in all six species. A total of 15 (DM 3, 8, 11, 13, 18, 27, 31, 39, 40, 45, 50, 52, 53, 54, & DM 55) and seven (DM13, 23, 24, 45, 48, 50 and DM53) produced unique bands in P. philippinensis and P. sparsa respectively. Representative examples of

Table 2: Examples of different types of microsatellite repeat structures identified in Peronosclerospora sorghi – pathotype. 3

| Microsatellite loci | Type                      | Repeat structures                           |
|---------------------|---------------------------|--------------------------------------------|
| DM2                 | simple and perfect repeat | GT GT GT GT GT GT GT                        |
| DM16                | simple and Imperfect repeat | TG TG TG TG TG TG TG TG TG TG TG TG TG TA TG TG TG TG TG TG TG TG TG TG TG TG TG TG TG T |
| DM13                | compound and imperfect    | CACACACACACACACACACACACACCCTGCTATATATA     |
Table 5: Amplification details of all 54 SSR loci in different downy mildew species

| Locus, Species, & Most related sequence (Genomic DNA/EST) | Query coverage (%) | Identity (%) | E-value* |
|----------------------------------------------------------|--------------------|-------------|----------|
| DM21 DQ45744, Phytophthora ramorum (Sudden oak death agent – Fungi), transposon GypsyPr-2 reverse transcriptase gene – Genomic DNA | 26 | 96 | 3e–20 |
| DM28 AF312890, Phytophthora cinnamomi SSR sequence – Genomic DNA | 45 | 90 | 4e–36 |
| DM53 DQ645744 – Phytophthora ramorum transposon GypsyPr-2 reverse transcriptase gene | 96 | 78 | 4e–70 |
| AY830104, (GypsyPi-3a), AY830105 (GypsyPi-3b) Phytophthora infestans – retrotransposon | 93 | 96 | 3e–14 |
| AF490339, Phytophthora infestans – Gypsy like retrotransposon, DQ645742 | 87 | 90 | 2e–09 |
| DM55 AY564217, Phytophthora ipomoeae | 78 | 90 | 4e–07 |
| EU427470.1 – P. ramorum | 54 | 90 | 6e–32 |
| AY564219 – P. andina, AY564218-P. phaseoli, AY564213 to AY564216 – P. mirabilis, AY564209 to AY003911 – P. infestans &DQ832717 and AY564221 | 54 | 89 | 3e–30 |
| - mitochondrion DNA | 54 | 86 | 1e–26 |
| ES287433 and ES286374Phytophthora brassicae | 54 | 88 | 2e–30 2e–29 |
| CV935202Phytophthora infestans (potato late blight) | 54 | 86 | 1e–26 |
| EL774547, EL777668, EL775494, EL779446, EL781147 and EL775427Phytophthora ultimum | 54 | 88 | 2e–30 2e–29 |
| - ESTs | 54 | 86 | 1e–26 |

*E value or the Expect value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size

The dendrogram constructed using UPGMA (Fig. 2) summarizes the interrelationship observed among 34 isolates of different downy mildew species. With the similarity coefficient greater than 0.84, all the 34 isolates were grouped into three distinct clusters. In the cluster I, 18 Peronosclerospora sorghi isolates including pathotype P3 (metalaxyl sensitive) and other metalaxyl resistant isolates of P3 and P6 pathotypes were all grouped together with a confidence limit of 80%. Within cluster I, Metalaxyl sensitive isolates of P1 pathotype (#1, 16 and 21 from Fig. 2)
were distinctly separated from others with a confidence limit of 79% and 76%. Five isolates of *P. maydis* and three isolates of *P. sacchari* were grouped in two sub-clusters with confidence limits of 83 and 74% respectively in cluster II and five isolates of *Sclerospora graminicola* were grouped in cluster III with confidence limit of 70%. The remaining *P. sparsa* and *P. philippinensis* isolates were not grouped into any of these three clusters and showed their distinct uniqueness from other species. Principal coordinate analysis (PCA) was also performed to show the relationship among the 34 isolates as a three dimensional display. In this analysis the first two principal components (having eigen value >1) explained 86% of the total variation. Like the UPGMA clustering dendrogram, PCA analysis placed isolates of different species of downy mildew into distinct positions (Fig. 3).

**Discussion**

The *Peronosclerospora* spp. that cause downy mildew of sorghum and maize and other gramineous hosts include some of the most destructive pathogens in the tropics and subtropics [36] and some of them infect more than one important crop. Of the 10 recognized species of fungi that cause downy mildew diseases of maize, 5 currently are in the genus *Peronosclerospora* [37,38]. The confusion over nomenclature of specific pathogens involved in these diseases is a major problem in global evaluation of the downy mildews caused by *Peronosclerospora*. It is difficult to determine if research results and disease control methods developed in one area of the world might be applicable to another. In several instances, taxonomy within the genus is confused because, at most, only slight differences in morphology of recognized species exist [39]. With the result of traditional approaches [40] combined with utilizing molecular techniques including isozyme analysis [41] and PCR (Polymerase Chain Reaction, [14]) the maize strain of *P. sorghi* from Thailand was given specific rank as *P. zeae* [13]. Legg [42] has outlined the advantages of some of the molecular methods for studying the *Peronosclerospora* spp. A maize strain of *P. sorghi* was reported in southern Nigeria [43]. However, Yao et al. [14] using PCR, were unable to differentiate an isolate of SDM from maize in southern Nigeria from sorghum/maize-infecting strains and the identity of this pathogen remains unknown. The problems described warrant development of simple and powerful molecular diagnostic tools for constant monitoring of this disease. Since microsatellite regions are highly mutable due to their propensity of addition and deletion of repeat copies they can be used to differentiate within and between related taxa, even at the level of individual isolates in a single species. Simple DNA-profiling methods based on microsatellite variability provide possibilities to identify individual genotypes for studies in population genetics, ecology and taxonomy [44].

**Characterization of microsatellite loci**

In the present study, a total of 55 microsatellite loci were isolated from *Peronosclerospora sorghi* – pathotype P3 DNA enriched for simple sequence motifs. We observed 33% internal redundant motifs, likely consequences of using two PCR amplification steps during the isolation process. Of the sequenced clones with an insert, over 79% contained microsatellites. This is a significant enrichment as compared to traditional microsatellite isolation protocol [45]. However, the relatively small fragment size average resulting from the use of three restriction enzymes in the initial fragmentation (HaeIII, (GGCC) targets, Rsal, (GTAC) and DraI (AAATTT)) may explain why 144 insert fragments out of 270 clones (53%) were truncated within, or at the end of, a microsatellite repeat. This result is in accordance with the CM collection [46], in which genomic DNAs were mechanically fragmented using a
high-pressure nebulizer, and a substantial portion of the clones were truncated within the microsatellite. The most frequent dinucleotide microsatellite motifs detected in our libraries were (CA/AC)$_n$ or (GT/TG)$_n$, followed by (GA/AG)$_n$ or (CT/TC)$_n$. In earlier studies, (CA)$_n$ repeat motifs [47] and (AG)$_n$ repeat motifs [48] were also detected from the ascomycetes, *Podospora anserina* and *Lobaria pulmonaria* respectively. Approximately 39% of all isolated loci were composed of complex intermixed motifs, which were rarely arranged in a tandem way. This pattern, known as cryptic simplicity [49], is common in larger eukaryotic genomes [50]. Microsatellites identified with trinucleotide repeats (CAA, AGG and ACA) were found in this study with low frequencies, but seem to differ from those typically found in 14 fungal genomes (AAG, AAC, AGC, and ATC) [51]. One trinucleotide repeat clone (DM45) included multiple repeats in a complex pattern but no significant homology was found to other sequences currently in GenBank.

Determining if microsatellites will be polymorphic can only be assessed empirically, however, two considerations can help predict if polymorphism is likely. First, as slippage during replication increases with the number of tandem repeats [52], loci in which the motif is iterated at least eight times are desirable. Second, it may be possible to predict the stability of a given microsatellite motif by assessing the likelihood of “expandability” [53]. Microsatellites reflect a balance between expansion and contraction, but while there is evidence of a bias toward increasing microsatellite size [54], very long tandem repetitions are rare [55]. In contrast, in our study, (CA/AC)$_n$
and (GT/TG)n were commonly repeated more than 20 times, suggesting expansion to these lengths is not limited. The presence of these motifs in most fungal and oomycete genomes suggests they would be useful microsatellites to target in different species where genome data are not available.

Retrotransposable Elements

The presence of retrotransposon-like elements (mobile genetic elements) plays an important role in genome evolution and such elements constitute about 5 to 10% of the genome in eukaryotes [56]. DM55 (ACA)19 showed significant sequence homology to mitochondrial sequences in several Phytophthora species (Table 3). Two of the P. sorghi SSR sequences were found to have a degree of similarity to reverse transcriptases, suggesting an origin from retrotransposons possibly present in a progenitor species. In this study, P. sacchari and S. graminicola isolates were not amplified with DM 21, whereas, DM53 cross amplified in all the species studied. These results suggest the GypsyPr-2 reverse transcriptase-like gene sequence as defined in Phytopthora ramorum was not present in P. sacchari or S. graminicola isolates. However, sequences similar to the Phytophthora infestans GypsyPi-3a and GypsyPi-3b retrotransposon-like and to the GypsyPs-1A sequence from Phytophthora sojae were present in all Peronosclerospora, Peronospora and Sclerospora downy mildew isolates. Primers and probes built to take advantage of the presence of unique differences in such retrotransposon sequences can be useful for strain/race identification. Examples include the work by Sastry et al. [57] with S. graminicola and Becker et al. [58] in the basidiomycete Chondrostereum purpureum.

Figure 3

Principal coordinate analysis (PCA). Three-dimensional display of 34 Peronosclerospora, Peronospora and Sclerospora isolates based on the combination of data obtained with 54 SSRs. Dimensions 1, 2 and 3 are accounted 86% of the variation observed.
Gel analysis
Resolving microsatellite amplicons in polyacrylamide gels using a LI-COR high throughput sequencing system with fluorogenic primers results in significantly more useful bands than resolving the same products in 4% SFR gels stained with ethidium bromide. For example DM12, 19, 30, and 38 gave monomorphic expression in SFR agarose gels but showed alleles with polymorphic differences in LI-COR poly-acrylamide gels. Taken together, from combined data across 34 Peronosclerospora, Peronospora and Sclerospora spp. isolates, an average of 4.8 polymorphic markers were detected per SSR in LI-COR poly-acrylamide gels versus 2.7 in SFR agarose gels. Thus the use of LI-COR poly-acrylamide gels with fluorogenic labeled primers was more efficient in separating bands which reflect clear cut polymorphisms. Fingerprinting using 54 SSR primer pairs over all the isolates resulted in 260 diagnostic alleles. Although it is assumed that the majority of size variants (100 to 350 bp) was due to variation in the number of the repeat motifs, this has not been verified by sequencing. Insertions, deletions and base substitutions in the flanking regions [59] might also account for variation in fragment length, especially when amplicons from different species are compared.

Cross-species amplification
It has been shown that microsatellite primers developed for a distinct species can be useful for genetic analysis in closely related species [60], but successful transferability depends upon the evolutionary distance between source and target species [61]. Dutech et al. [62] reported relatively low cross-species transferability of microsatellites in fungi. Within genera, only 34% of the loci tested could be transferred, which appears much lower than in animals or plants. Here, twelve SSRs (DM 9, 12, 13, 14, 16, 19, 30, 31, 35, 49, 53 and DM54) were cross amplified in samples from 6 species of downy mildew-causing oomycetes. Successful cross-amplification simply indicates that the flanking regions are conserved across the species studied, but it does not tell anything about the character and structure of the fragment and additional sequencing will be necessary to clarify the structure of these fragments. Similar findings with successful cross amplification of SSRs in closely related species were reported for lichen-associated fungi [48] and in for a variety of fungal species [51]. Another interesting feature evident from our study is the distinctness of different species as revealed by unique banding patterns with different SSRs as evinced clearly from Fig. 1. Unique bands were noted with DM 3, 8, 11, 13, 18, 27, 31, 39, 40, 45, 50, 52, 53, 54, & DM 55 for P. philippinensis and DM13, 23, 24, 45, 48, 50 and DM53 for P. sparsa. While these amplicons may be useful for diagnostics based on unique banding patterns, the information for P. philippinensis and P. sparsa is based on single isolates, so data from more independent, species-verified samples will be required to assess the degree of variation within natural populations. Based on the species-specific clustering of isolates where multiple samples were available, distinctive fingerprints can be anticipated.

Cluster analysis
In the present study, SSR markers revealed using high throughput LI-COR-NEN® Model 4300 -dual-dye automated DNA Analyzer have been used to resolve cryptic genetic variation of closely related different downy mildew species that have been impossible to resolve with morphological systematic characters. SSR fingerprinting was conducted using 54 primer pairs and genomic similarity analyses derived from qualitative data grouped isolates of samples of downy mildew according to host specificity (sorghum, maize, sugarcane, pearl millet and rose) whose taxa had been uncertain based on morphological criteria. In this study, metalaxyl sensitive P3 isolate (#2 in Fig. 2) in cluster I grouped with all other metalaxyl resistant isolates, suggested that metalaxyl resistance may have originated in a P3 isolate to give rise to the recent outbreak, consistent with results of based on AFLP molecular characterization [15]. However, mating and recombination involving metalaxyl-resistant genotypes and other isolates, along with selection in favor of resistance would narrow the genetic base and can account for the high similarity (approximately 89%) among all 19 P. sorghi isolates in cluster I. P. maydis and P. sacchari isolates in cluster II were grouped into two sub clusters with high similarity coefficient (approx. 82%) indicates that corn and sugarcane downy mildew isolates have narrow genetic differences, showing more genetic similarities with each other than other Peronosclerospora species. The single isolates of P. philippinensis and P. sparsa were not grouped with any of the three clusters and showed their uniqueness due to their distinct banding pattern with different microsatellite primer combinations as detailed earlier. The three-dimensional comparison is compatible with cluster analysis and provides a good visual comparison of the genetic similarities and differences of the isolates. Fig. 3 clearly depicted the unique identity of P. philippinensis from other species. This display shows that the genetic distance between P. philippinensis and P. sacchari is very narrow. Similar findings with close phylogenetic relationship was recorded through isozyme analysis between P. philippinensis and P. sacchari isolates from Taiwan [26,63] and by RFLP patterns by [13]. Further study is clearly needed to confirm this relationship. Unfortunately, the select agent status of P. philippinensis has made the obtaining of additional samples, even of DNA, impossible to date.

Conclusion
Microsatellite primer sets developed from P. sorghi sequences proved to be useful for all downy mildew species analyzed and are likely to be increasingly developed...
and applied to studies of pathogen epidemiology, population biology, and genomics. The diversity of microsatellite motifs gave each species a unique "signature" of repeat distributions. Subsets of the 54 newly developed SSRs may be very useful for rapid and efficient identification and genetic analysis of the natural populations and host range of these obligate oomycetes. The unique banding pattern of P. philippinensis from fifteen (DM 3, 8, 11, 13, 18, 27, 9, 39, 45, 50, 52, 53, 54, & DM 55) and P. sparsa from seven (DM13, 23, 24, 45, 48, 50 and DM53) SSRs make these primers useful as diagnostic markers for the respective species. In future studies it will be possible to focus on the population diversity and recombination within and between native and introduced populations of the closely related species. These distinct fingerprinting profiles can be used as diagnostic tools to formulate breeding strategies targeting host resistance to local pathogens and for monitoring the emergence of new virulent races.

Authors' contributions
RP performed the capturing of microsatellites, data analysis and wrote the manuscript. PN and UKR provided lab facilities and assisted in capturing microsatellites. RP and SRE carried out all gel analyses. EGN performed sequence analysis. LKP, GNO, DGL and CWM provided the source materials. DGL and CWM directed and oriented the project and revised the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Information on 55 SDM (Peronosclerospora sorgi) microsatellites loci. Microsatellite loci repeat motif, primer sequence (forward and reverse), allele size, annealing temperature (Tm), number of alleles detected and gene bank accession numbers.
Click here for file.
[http://www.biomedcentral.com/content supplementary/1471-2156-9-77-S1.xls]

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