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FULL RESEARCH PAPER

Differentiation of two powdery mildews of sunflower (*Helianthus annuus*) by a PCR-mediated method based on ITS sequences

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Abstract Powdery mildew can be found in most sunflower fields during the winter season in Taiwan and causes severe yellowing on the blade, petiole, stem, and calyx, as well as serious defoliation. Two types of powdery mildew fungi isolated from sunflower leaves showed variable status for fibrosin bodies. But only the cleistothecium of *Podosphaera xanthii*, one of the pathogens causing this disease, was observed on samples from Chungpu County at the beginning of 2005. With a species-specific primer pair, PN23/PN34, no specific PCR product was amplified from the pathogen's genomic DNA. Based upon the ITS sequence of rDNA, three PCR primer sets (S1/S2, G1/G2, and L1/L2) specific to *P. xanthii*, *Golovinomyces cichoracearum* and *Leveillula taurica*, respectively, were designed to detect and differentiate pathogens causing powdery mildews on sunflower. Only the primer pairs S1/S2 and G1/G2 could amplify PCR products, with product sizes of 454 and 391 bp, respectively. Four samples of fungal DNA were subjected to a multiplex PCR amplification with primer pairs S1/S2 and G1/G2; *P. xanthii* and *G. cichoracearum* were successfully detected. These results suggest that the multiplex PCR method is a rapid, simple, and effective technique to detect and differentiate powdery mildews, for example *P. xanthii* and *G. cichoracearum*, found on sunflower. With morphologic characteristics, ITS sequence analysis and pathogenicity testing, *P. xanthii* and *G. cichoracearum*, the first case, are two powdery mildews on sunflower in Taiwan.

Keywords Multiplex PCR · Polymerase chain reaction · Powdery mildew · Sunflower

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Introduction

Sunflower (*Helianthus annuus*) is a native plant of North America (Heiser 1954) and has recently been used for oil production. Since 1969, sunflowers have been introduced from Canada, South Africa, and the USA by the Tainan District Agricultural Improvement Station (TDAIS) of Taiwan for its oil production programme. Due to disease problems such as powdery mildew, *Sclerotinia* basal stalk rot, and *Botrytis* head rot, cultivating sunflowers for economical oil

production is no longer feasible (Fang 1973) and the plants are raised primarily for use as a green manure. However, TDAIS research into collecting and breeding sunflowers is still progressing for cut-flower, houseplant, landscaping, or green manure purposes (Hsieh 2002).

In sunflowers, the first sign of powdery mildew usually appears as primarily white mildew on the infected leaves in September; the mildew becomes severe on all aerial parts of the plant under heavy infection during the blooming stage of the plant (Fang 1973). During late stages of the infection, superficial mycelia may enlarge and merge until most of the plant surface is covered. At least three powdery mildew genera, including *Golovinomyces cichoracearum* (= *Erysiphe cichoracearum*) and *Leveillula taurica* (Saliman et al. 1982; Yang et al. 1988; Gulya et al. 1991; American Phytopathological Society 1994; Braun 1995), have been reported. In Taiwan, one of the most serious diseases in sunflower is powdery mildew caused by *Podosphaera xanthii* (= *Sphaerotheca fuliginea*) (Fang 1973). Classification of these powdery mildews is needed. However, classical identification methods are labour-intensive and require considerable experience in differentiating the morphologies of these powdery mildews (Grote et al. 2002). Moreover, the fact that some powdery mildew fungi produce sexual spores or have a non-sexual stage at the end of the growing season has complicated the taxonomic characterization of these pathogens (Kashimoto et al. 2003). However, differences in the sequence of nucleotides in internal transcribed spacer (ITS) regions of rDNA and PCR using species-specific primers have been used very effectively for detecting or identifying powdery mildews (Takamatsu and Kano 2001; Cheng et al. 2006; Shi and Mmbaga 2006).

In diseased sunflower leaves, conidia ellipsoidal in shape and in chain structures similar to the conidia of either *Podosphaera* or *Golovinomyces* were observed (Braun et al. 2002). However, some conidia contained crystal-like inclusions, so-called fibrosin bodies, which are characteristic of the genus *Podosphaera*, and other conidia lacked fibrosin bodies. This implies the existence of more than one powdery mildew on sunflowers in Taiwan. For rapidly identifying and differentiating powdery mildew fungi other than *P. xanthii*, we designed specific primers based upon the ITS sequence of rDNA from GenBank of NCBI to detect three possible pathogens by a PCR-mediated method and developed a multiplex PCR method to detect and differentiate powdery mildews found on sunflower.

Materials and methods

Sample sources

Leaves of sunflowers infected with powdery mildews were collected from fields in Chungpu County, Chiayi City, and Putze City in January, 2005. Fungal species were identified from diseased plant samples in the laboratory. The length and width of 30 conidia from each field were measured. The presence of fibrosin bodies, vacuoles and the type of germination tube of the fungal structures associated with the anamorph of powdery mildew fungi were recorded. Cleistothecia, asci, ascospores of the fungal structures associated with the teleomorph of powdery mildew fungi, and the appendages on the surfaces of the cleistothecia were also recorded. Four samples from three counties as mentioned above were collected for the PCR and the multiplex PCR reactions. *Podosphaera xanthii* and *G. cichoracearum* were identified by morphological characters and molecular detection. Powdery mildews growing on twenty leaves were collected in a commercial plot in Chungpu County in 2006. These samples were amplified by PCR with primer pairs S1/S2 and G1/G2. For the PCRs, reference isolates – powdery mildews of *Physalis angulata* (*P. xanthii*), gerbera (*P. xanthii*), melon (*P. xanthii*), carrot (*Erysiphe heraclei*), *Murraya paniculata* (*Oidium murrayae*), vegetable soybean (*Erysiphe diffusa*), *Mentha canadensis* (*Golovinomyces biocellatus*), and *Capsicum annuum* (*L. taurica*) – were used.

Pathogenicity test

Podosphaera xanthii and *G. cichoracearum* isolates with or without fibrosin bodies in the conidia were used for pathogenicity tests by touching healthy plants (21 day-old cv. Tainan No.1) to leaf samples bearing conidia; non-inoculated plants served as controls. The experiment was performed twice with six plants per treatment (three pots with two plants per 5 l pot). After inoculation, different treated plants were maintained separately in different rooms of a

greenhouse at 25°C under natural daylight conditions. Powdery mildew symptoms were assessed 7 days after inoculation.

DNA extraction, primers, and PCR amplification conditions

Mycelia scraped from diseased leaf tissues were transferred into a 2 ml microfuge tube and then ground into a powder with a plastic pestle immediately after freezing in liquid nitrogen. The procedure for DNA extraction and purification was modified from a previously described protocol (Takamatsu and Kano 2001). After the addition of 700 µl lysis buffer (50 mM Tris-HCl, pH 7.2; 50 mM EDTA, pH 7.2; 3% SDS; 1% mercaptoethanol), the mycelial solution was vortexed and heated in a water bath at 65°C for 1 h. Next, the DNA solution was extracted and mixed well with 700 µl of phenol/chloroform, and then the mixture was centrifuged at 12,000 x *g* for 10 min. The upper, aqueous phase was then mixed with 500 µl of chloroform and centrifuged at 12,000 x *g* for 4 min. For DNA precipitation, 500 µl of the aqueous phase were transferred into a new 1.5 ml eppendorf tube; then 50 µl of 3 M sodium acetate (NaOAc) and 500 µl of isopropanol were added. The DNA was pelleted at 12,000 x *g* for 20 min. The DNA pellet was washed with 500 µl of 70% ethanol and then centrifuged at 12,000 x *g* for 20 min. After air-drying, the DNA pellet was dissolved in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 200–500 µg ml^{-1}.

The internal transcribed spacer (ITS) of nuclear ribosomal DNA regions was amplified from powdery mildew of sunflower using the powdery-mildew-specific ITS universal primer pair PN23 (5'-CAC CGC CCG TCG CTA CTA CCG-3')/PN34 (5'-TTG CCG CTT CAC TCG CCG TT -3') (Bardin et al. 1999). The PCR reaction mixture contained 0.15 mM dNTPs, 0.4 µM primers, 1 U Taq Polymerase (BioBasic), 1X PCR buffer with 1.5 mM $MgCl_2$, and 10 µg of template DNA. Sterile, distilled water was added to reach the final volume of 25 µl. PCR amplification was carried out with a thermal cycler (Eppendorf Mastercycler personal, Eppendorf Scientific, Westbury, NY) using the following procedure: 5 min at 94°C for the initial denaturation, followed by 30 cycles consisting of 40 s of denaturation at 94°C, 1 min of annealing at 62°C, and 1.5 min of DNA synthesis at 72°C. A final extension time of 5 min at 72°C was added at the end of the last cycle. Five microliters of PCR products were separated by gel electrophoresis on a horizontal 2% agarose gel. The gel was stained with ethidium bromide (0.5 µg ml^{-1}) and DNA fragments were visualized and photographed under UV light.

Pairs of primer, S1 (5'- GGA TCA TTA CTG AGC GCG AGG CCC CG -3')/S2 (5'- CGC CGC CCT GGC GCG AGA TAC A -3'), G1 (5'- TCC GTA GGT GAA CCT GCG GAA GGA T -3')/G2 (5'- CAA CAC CAA ACC ACA CAC ACG GCG -3'), and L1 (5'- CCC TCC CAC CCG TGT CGA CTC GTC TC -3')/L2 (5'- CTG CGT TTA AGA GCC GCC GCG CCG AA -3'), that were specific to the ITS regions of *P. xanthii*, *G. cichoracearum*, and *L. taurica*, respectively, were designed based upon the ITS sequence of rDNA obtained from GenBank at the National Centre for Biotechnology Information (NCBI), i.e. AB040332 (Hirata et al. 2000), AY739110, and AB044378 (Khodaparast et al. 2001). The specificity of these primer pairs was analyzed by using related fungal species as mentioned above; the PCR reaction mixtures and PCR amplifications were as described above. PCR products were extracted with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and the purified DNAs were then sequenced by using an automated sequencer at the Minsheng Biotechnology Co. (Taipei, Taiwan).

Detection of *P. xanthii* and *G. cichoracearum* on diseased sunflower by PCR and multiplex PCR

Twenty diseased sunflower leaves were randomly and diagnostically collected from a field in Chungpu County. Mycelia collected from a single diseased leaf were transferred into a new 2 ml microcentrifuge tube and their total genomic DNA was extracted and purified using the method described above (Takamatsu and Kano 2001). Primer pairs S1/S2 and G1/G2 were used to detect *P. xanthii* and *G. cichoracearum*. Next, four fungal DNA samples were collected from sunflower fields in Chungpu County, Chiayi City, and Putze City. The DNA was dissolved in 50 µl of TE buffer and 5 µl aliquots of template DNA were used in multiplex PCR amplifications with primer pairs S1/S2 and G1/G2 simultaneously to detect and differentiate *P. xanthii* and *G. cichoracearum*.

Results

Morphology identification and pathogenicity test of powdery mildews

Podosphaera xanthi cleistothecia, 56–80×56–70 μm in size, were observed on diseased samples from Chungpu County. Each cleistothecium contained a single, globular ascus with the dimensions 52–70×44–56 μm, and each ascus accommodated eight elliptical ascospores, 12–20×10–16 μm in size. In addition, most ellipsoid conidia in chain formation with sizes of 20–48×16–20 (33.5×17.9) μm contained fibrosin bodies (Fig. 1a).

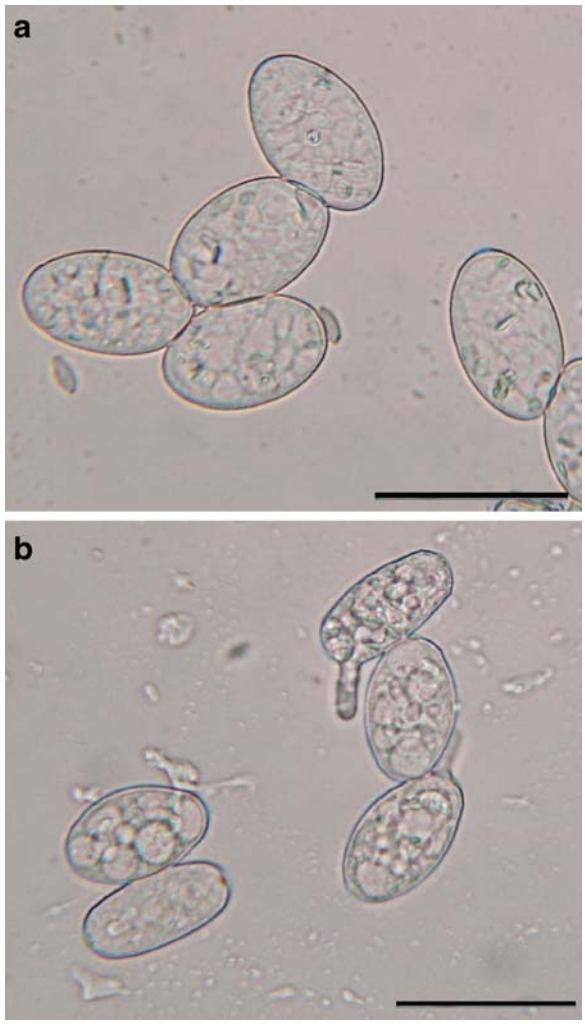

Fig. 1 Two powdery mildew fungi found on sunflower leaves. **a** Fibrosin bodies in mature conidia of *Podosphaera xanthii*. **b** Spores of *Golovinomyces cichoracearum* lacking fibrosin bodies and a germinated conidium of *G. cichoracearum* that has formed a simple germ tube (scale bar: 40 μm)

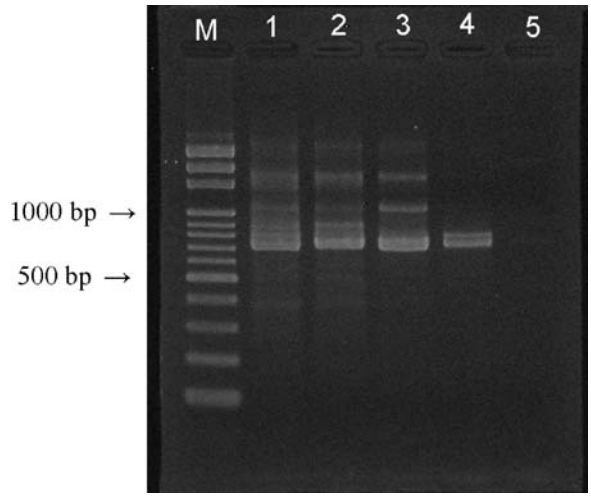

Fig. 2 PCR products, from DNA templates extracted from four diseased sunflower leaves collected at three locations, amplified by using primer pair PN23/PN34 to target powdery mildew. M, 100 bp marker; Lane 1: Chungpu County A; Lane 2: Chiayi City; Lane 3: Putze City; Lane 4: Chungpu County B; and Lane 5: negative control

Chain and ellipsoid conidia of another powdery mildew on diseased samples from Chungpu County were 20–48×16–20 (29.7×17.2) μm in size and contained no fibrosin bodies (Fig. 1b). All conidia germinated well to form a long, simple germ tube. After 7 days of infection, *P. xanthii* or *G. cichoracearum* induced typical powdery mildew symptoms on the inoculated plants. As usual, no symptoms were observed on the leaves of non-inoculated plants.

Characteristics of the ITS region of *P. xanthii* and *G. cichoracearum*

Although the known universal primer pair PN23/PN34 was used to amplify PCR products from the powdery mildew, non-specific products were always amplified from fungal DNA (Fig. 2), suggesting a new primer set is needed to detect specific powdery mildews. Based on the sequence variation in the ITS region, species-specific primer pairs were designed: S1/S2, G1/G2, and L1/L2 specific for *P. xanthii*, *G. cichoracearum*, and *L. taurica*, respectively (Fig. 3). A 454 bp product amplified by the primer pair S1/S2 was observed from mildews sampled from sunflower, *Physalis angulata*, gerbera, and melon, but not from mildews on carrot (*E. heraclei*), Murraya

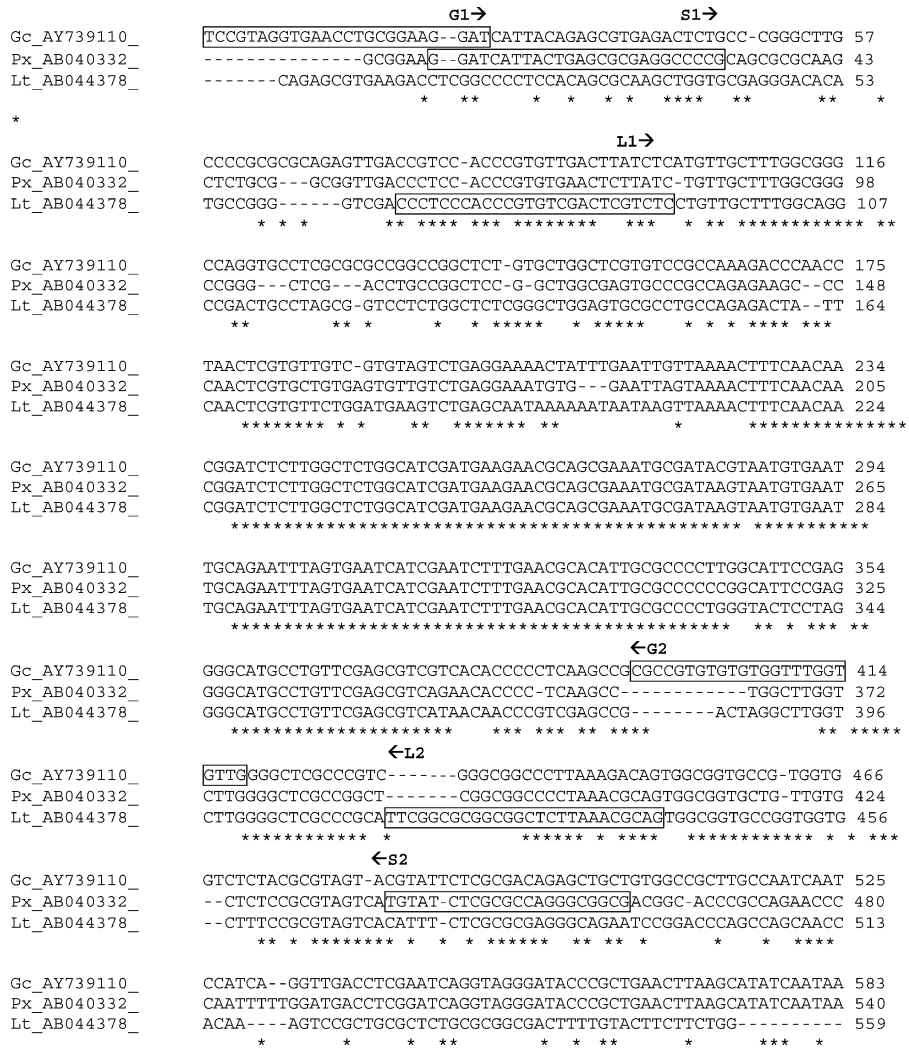

Fig. 3 Nucleotide sequence comparison of the internal transcribed spacer 1 (ITS1), 5.8S, and ITS2 regions of *Golovinomyces cichoracearum* (AY739110), *Podosphaera xanthii* (AB040332), and *Leveillula taurica* (AB044378). The locations of polymerase chain reaction (PCR) primers for *G. cichoracearum*, *P. xanthii*, and *L. taurica* are delimited by boxes. The 5′ to 3′ direction of the PCR primers is shown by *arrows*

(*O. murrayae*) or soybean (*E. diffusa*) (Fig. 4a). Sequence analysis (EF010913 and EF010914) of this PCR product showed a 1% nucleotide difference compared with the PCR products amplified from *P. xanthii* (formerly known as *P. fusca*.) collected from sunflower (NCBI accession no. AB040311-12), cucurbits (NCBI accession no. AB040324), and *Youngia denticulata* (NCBI accession no. AB040351). Using primer pair G1/G2, a 391 bp DNA fragment was amplified from samples of sunflower powdery mildew, but not from others as described above (Fig. 4b). There was 100% nucleotide identity in the ITS1-5.8S-ITS2 region for the mildew from sunflower examined in this study and accession no. AY739110 for *G. cichoracearum*. With primer pair L1/L2, no PCR product was amplified from sunflower powdery mildew, although a specific product of 374 bp appeared for the mildew sample of pepper (*L. taurica*).

Detection of *P. xanthii* and *G. cichoracearum* on sunflower by PCR and multiplex PCR

With PCR testing of powdery mildews from 20 leaves with primer pairs S1/S2 and G1/G2, the prevalence of *P. xanthii* and *G. cichoracearum* was 50 and 100% respectively. Next, when multiplex PCR amplification with primer pairs S1/S2 and G1/G2 together was performed for fungal DNA samples from the diseased sunflowers in Chungpu County, Chiayi City, and Putze City, *P. xanthii* and *G. cichoracearum* were successfully and distinguished and detected (Fig. 5). Among the samples tested, *G. cichoracearum* was

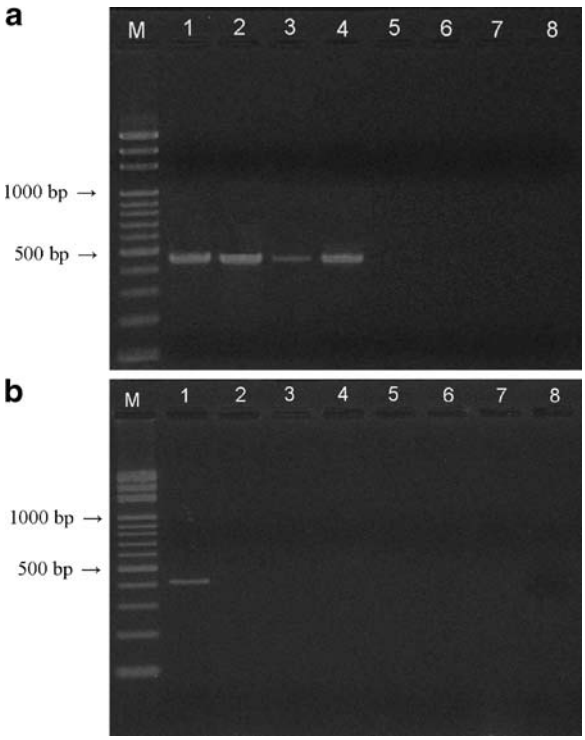

Fig. 4 Electrophoretic profile of PCR products amplified by primers S1/S2 (**a**) and G1/G2 (**b**). PCR products were separated by electrophoresis through a 2% agarose gel. M: 100 bp marker. **a** Specific PCR detection of *Podosphaera xanthii* from field samples of sunflower and other host plants. Lane 1: *P. xanthii* (*Helianthus annuus*, Chungpu County); Lane 2: *P. xanthii* (gerbera); Lane 3: *P. xanthii* (melon); Lane 4: *P. xanthii* (*Physalis angulata*); Lane 5: *Erysiphe heraclei* (carrot); Lane 6: *Erysiphe diffusa* (vegetable soybean); Lane 7: *Oidium murrayae* (*Murraya paniculata*); and Lane 8: negative control. A fragment of the expected size (454 bp) is observed in lanes 1, 2, 3, and 4. **b** Specific PCR detection of *Golovinomyces cichoracearum* from field samples of sunflower and other host plants. Lane 1: *G. cichoracearum* (*Helianthus annuus*, Chungpu County); Lane 2: *P. xanthii* (gerbera); Lane 3: *P. xanthii* (melon); Lane 4: *Erysiphe heraclei* (carrot); Lane 5: *Erysiphe diffusa* (vegetable soybean); Lane 6: *Oidium murrayae* (*Murraya paniculata*); Lane 7: *Golovinomyces biocellatus* (*Mentha canadensis*); and Lane 8: negative control. A fragment of the expected size (391 bp) is observed in lane 1

detected in all samples, whereas *P. xanthii* was only found in three samples.

Discussion

Since powdery mildews are diverse, molecular phylogenic analysis of ITS sequences has been employed to confirm host specificity (Hirata et al. 2000). A phenomenon has been reported in which powdery mildews on cosmos and cucumber have adapted to their hosts both genetically and pathogenically and therefore species-specific powdery mildews cannot cause cross-infection in Japan (Hirata and Takamatsu 2001). Earlier, powdery mildew of sunflower caused by *G. cichoracearum* and *L. taurica* had been reported in different areas around the world (Saliman et al. 1982; American Phytopathological Society 1994; Braun 1995). Here, two powdery mildew fungi on sunflower were examined in greater detail. As one of the more important powdery mildew fungi, *P. xanthii* infects more than 133 plant species in Taiwan (Hsieh 1983; Kuo et al. 1991; Cheng et al. 2006). With morphological differences in the formation of cleistothecia, such structures were restricted to samples collected from Chungpu County and did not appear either in samples from two other places in 2005 or in any samplings in 2006, suggesting that the appearance of cleistothecia is influenced by location and year.

The present of refractive fibrosin bodies in conidia are characteristic for identifying the *Oidium* state of *P. xanthii* (Reifschneider et al. 1985). However, conidia did not reveal well-developed fibrosin bodies and this deficiency increased the difficulty of identifying fungi as *P. xanthii* and/or *G. cichoracearum* on the basis of the morphology of the anamorph stage (Fig. 1a,b) when they coexisted on the same leaf.

Therefore, highly interspecific variations of ribosomal internal transcribed spacer (ITS) regions pro-

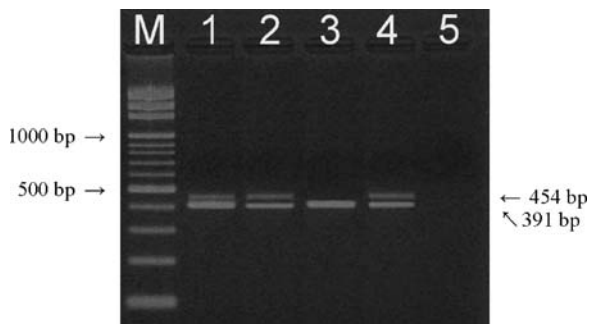

Fig. 5 Analysis of DNA fragments amplified by a multiplex PCR from DNA templates extracted from four diseased sunflower leaves collected at three locations. Two primer pairs (S1/S2 and G1/G2) for *Podosphaera xanthii* and *Golovinomyces cichoracearum*, respectively, were used, and the PCR products were separated on a 2% agarose gel. M, 100 bp marker; Lane 1: Chungpu County A; Lane 2: Chiayi City; Lane 3: Putze City; Lane 4: Chungpu County B; and Lane 5: negative control

vide effective targets for phylogeny studies as well as the molecular detection of fungi (Hirata and Takamatsu 1996; Takamatsu and Kano 2001). The universal ITS primers (White et al. 1990) and powdery-mildew-specific primers PN23/PN34 (Bardin et al. 1999) were designed. However, non-specific PCR products always appeared (Fig. 2). Therefore, three PCR primer pairs (S1/S2, G1/G2, and L1/L2) specific for *P. xanthii*, *G. cichoracearum*, and *L. taurica* were designed and used to differentiate pathogens causing powdery mildews on sunflowers (Figs. 3, 4a,b). In Taiwan, *G. cichoracearum* was first reported to cause the disease on sunflower. The PCR-based, molecular method required less labour and time than did microscopic methods or spore trapping (Patzak 2005). The PCR method was found to be accurate and sensitive and can be used for routine detection and classification, as well as disease surveillance. Nevertheless, multiplex PCR is a more valuable diagnostic tool for the rapid detection of several pathogens in host plants (Fraaije et al. 2001; Patzak 2005). Therefore, a rapid multiplex-PCR-based detection method was developed to effectively identify and differentiate *P. xanthii* (50%, 10/20) and *G. cichoracearum* (100%, 20/20) directly from total DNA extracts of infected sunflower plants (Fig. 5). Considering the limitations of small sample sizes, this specific detection method will be further evaluated on a large scale to survey *P. xanthii* and *G. cichoracearum* in sunflower plants.

In sunflower, *G. cichoracearum* was a more prevalent pathogen than *P. xanthii* (50%) in causing powdery mildew. In contrast, *P. xanthii*, with short-lived and wind-dispersed conidia, infects many other plants; *G. cichoracearum* infects only a few host plants such as *Sonchus oleraceus*, *Mallotus japonicus*, and *Dahlia pinnata* in Taiwan (Kuo et al. 1991). Both *P. xanthii* and *G. cichoracearum* infect cucurbits separately in central and southern Taiwan (Huang 1992; Tsay and Tung 1992) and *G. cichoracearum* was found in 9–39% of the mildewed leaf samples of cucurbits in France (Bardin et al. 1999) and differs from *P. xanthii* in pathogenicity against certain cucurbit cultivars (Epinat et al. 1993). Given the difference in infection rate between these two pathogens, *G. cichoracearum* and *P. xanthii*, the multiplex PCR method developed in this study provides a valuable diagnostic tool to differentiate these powdery mildews and quickly identify the coexistence of these two pathogens on the same host plant tissue.

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