Phellinus noxius: molecular diversity among isolates from Taiwan and its phylogenetic relationship with other species of Phellinus based on sequences of the ITS region

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Abstract

Background: Analysis of phylogenetic relationship of 91 isolates of Phellinus noxius obtained from 46 plant species in Taiwan did not show distinct grouping based on ITS sequences.

Results: However, the ITS nucleotides showed 20 different kinds of variations including single nucleotide polymorphisms, deletion and insertion in ITS1 and ITS2, but none in 5.8 S. The Taiwanese isolates of P. noxius were dividable into long (type L), median (type M) and short (type S) groups based on ITS sequence length. Two isolates with identical ITS sequence belonged to types L. Type M with 72 isolates was further divided into 33 subtypes, while types S with 17 isolates was further divided into two subtypes.

Conclusion: Phylogenetic analysis of ITS sequences among Phellinus species showed that isolates of P. noxius were in the same clade distinctly separated from other Phellinus species.

Keywords: Deletion, Insertion, ITS sequence, Nucleotide variation, Sequence length, Single nucleotide polymorphism

Background

Brown root rot caused by Phellinus noxius (Corner) G. H. Cunn. is widespread among tropical countries in Southeast Asia, Africa, Oceania, Central America and the Caribbean (Pegler and Waterston 1968). In China, it has been reported from the tropical Hainan Island (Tai 1979). In Japan, it was found on the subtropical island of Okinawa (Abe et al. 1995). The pathogen attacks more than 120 species of fruit and ornamental trees in both tropical and subtropical districts in Taiwan (Ann et al. 1999; Chang and Yang 1998). Among the approximately 200 plant species listed as hosts of P. noxius in the world, about half of them were reported for the first time from Taiwan (Ann et al. 2002). Even though the fungus lacks air-borne spores for efficient dissemination, it is very widespread and occurs on so many kinds of hosts at very different geographic locations on the island of Taiwan (Ann et al. 2002). It is, therefore, conceivable that P. noxius may be an ancient residence of the island where diverse isolates of this fungus may have existed. There are very few morphological characters in P. noxius available for testing this hypothesis because the fungus rarely produces basidiocarps on diseased trees in the fields (Ann et al. 1999; Chang 1995, 1996).

In this study, molecular variation in the ITS (ITS1, 5.8S and ITS2) region among isolates of P. noxius from Taiwan was investigated and compared with the ITS sequences reported from other countries available in the GenBank. We also investigated the ITS phylogenetic relationship of
**P. noxius** with other species of *Phellinus*. Details of the study are reported herein.

**Methods**

**Isolation and storage of the pathogen**

Main roots of trees showing quick or slow decline symptoms (Ann et al. 2002) were exposed and examined. Those showing typical brown discoloration were cut and brought back to the laboratory. Small pieces (5 × 2 × 1 mm) of tissue were obtained from the advancing margins of the diseased roots, surface-sterilized with 0.5% NaClO for 1 min, plated on potato dextrose agar (PDA) supplemented with 100 ppm streptomycin sulfate and 10 ppm benomyl for inhibition of growth of bacteria and other fungi, and incubated at room temperature (24–30 °C). Fungal mycelia growing from diseased tissue were transferred to 2% water agar. Single-hyphal tips obtained from the fungus growing on water agar were cultured on PDA and stored in sterile distilled water in test tubes at room temperature (Boesewinkle 1976; Ko 2003). From each diseased tree only one isolate was saved for the study. The cultures were identified as *P. noxius* based on the production of brown colonies with irregular dark brown zone lines on PDA and formation of arthrospores and trichocysts (Ann and Ko 1992).

DNA extraction, amplification and sequencing

Each isolate of *P. noxius* was grown on cellophane placed on PDA (Ko et al. 2011). After incubation at 25 °C for 10 days, mycelia were harvested, lyophilized and stored at −20 °C until use. About 20 mg lyophilized mycelia were ground in liquid nitrogen and used for extraction of DNA using the genomic DNA extraction kit (GenMark Technology Co., Taichung, Taiwan). The ITS (ITS1-5.8S-ITS2) region was amplified with primer pair of ITS4 and ITS5 (White et al. 1990). The 25 μl reaction mixture consisting of 0.2 μg template DNA, 0.2 μM each primer, 200 μM each dNTP, 2 μl 2X polymerase chain reaction (PCR) buffer and 1.0 U ZyM Taq DNA polymerase (Zymeset, Taiwan) was subjected to thermal cycling in a Perkin-Elmer Thermal Cycler 9700 (Perkin-Elmer Applied Biosystem, USA). Cycling conditions for amplification were an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 45 s, 72 °C for 45 s, and a final elongation at 72 °C for 7 min. The PCR products were electrophoresed on a 1.5% agarose gel. Direct sequencing of the PCR products was performed by the Seeing Bioscience Company (Taipei, Taiwan), using ITS4, ITS5 (White et al. 1990), PN-5.8S-1 (5′-GCA GCG AAA TGC GAT AAG TA-3′), or PN-5.8S-2 (5′-CAT GAC ACT CAA ACA GGC AT-3′) as the primer.

The sequences of ITS region obtained from the sequencing process were assembled, trimmed and edited using the Vector NT1 software v. 10.0 (InforMax Inc., USA). The sequence of ITS tail was determined using the ITS 2 annotation tool (Keller et al. 2009). The polymorphic portions were marked by IUPAC ambiguity codes. The ITS sequences of 36 isolates of *P. noxius*, representing all ITS types found in Taiwan, were submitted to NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

**Phylogenetic analysis**

The ITS sequences of 91 isolates of *P. noxius* from Taiwan were analyzed in order to understand the phylogenetic relationship among these isolates. Multiple alignments and minor adjustments of the sequences of these isolates were performed using clustal X 1.81 (Thompson et al. 1997) followed by BioEditor software. Sequence alignment was deposited at TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:516384). Phylogenetic relationships were analyzed using the Philip 3.67t software (Phylogeny Inference Package, Version 3.67) and the neighbor joining program with 1000 bootstrap replicates. The program of Treeview was used to view phylogenetic trees.

In order to study the phylogenetic relationship between isolates of *P. noxius* from Taiwan and other countries and other *Phellinus* species, the ITS sequences of all *Phellinus* species in the GenBank were retrieved. A total of 58 isolates belonging to 39 species of *Phellinus* was obtained and used for phylogenetic analysis (Table 1). The ITS types L, M and S divided based on ITS length were used as local strains for analysis. The method described above was used for phylogenetic relationship analysis.

**Results**

Phylogenetic relationship among Taiwanese isolates of *P. noxius*

A total of 91 isolates of *P. noxius* was obtained from 46 species of plants distributed in different geographic locations in Taiwan from 1991 to 2009 (Table 2). Analysis of the phylogenetic relationship of these Taiwanese isolates did not show distinct grouping based on ITS sequences. The bootstrap values on the branches were very low and were all below 50% (data not shown) with accession number JN836346-JQ003229 (Tables 1, 2).

Nucleotide variation in ITS region among Taiwanese isolates of *P. noxius*

The examination of ITS nucleotide variation revealed the existence of 20 different kinds of variants, designated...
Table 1  List of taxa of *Phellinus noxius* and related species from GenBank used for phylogenetic analysis

| Species          | Geographic origin | Strain no. | ITS sequence length (bp) | Accession no. |
|------------------|-------------------|------------|--------------------------|---------------|
| 1. *Phellinus alni* | TW322             | 610        |                          | AY340041      |
| 2. *P. badius*    | CBS 449.76        | 663        |                          | AY558609      |
| 3. *P. baumii*    | MPNU 7006         | 711        |                          | AF200231      |
| 4. *P. bicuspidatus* | KCTC 6651    | 621        |                          | AY558610      |
| 5. *P. calcitratus* |                 | 584        |                          | JF894115      |
| 6. *P. chrysosoma* |                  | 644        |                          | AF055370      |
| 7. *P. cinereus*  | OS-37             | 620        |                          | AM931248      |
| 8. *P. conchatus* | CBS 167.29        | 708        |                          | AY558614      |
| 9. *P. ferrugineovelutinus* | CBS 218.48 | 542        |                          | AY558618      |
| 10. *P. gilvus*   | ATCC26729         | 613        |                          | AF250932      |
| 11. *P. hartiï*   | CBS 162.30        | 692        |                          | AY558621      |
| 12. *P. l. p. paehoeicola* | CBS 252.50   | 705        |                          | AY558622      |
| 13. *P. igniarius* | CFMR 5609       | 609        |                          | AY558623      |
| *P. igniarius*    | KCTC6228          | 598        |                          | AF056192      |
| 14. *P. igniarius* var. *trivialis* | CBS 512.63   | 596        |                          | AY558624      |
| 15. *P. johnsonianus* | ATCC60051    | 702        |                          | AF250931      |
| 16. *P. laevigatus* | CFMR 5640       | 588        |                          | AY558626      |
| 17. *P. linteus*  | MPNU 7002         | 670        |                          | AF200228      |
| 18. *P. lundelii* | CBS 540.72        | 605        |                          | AY558630      |
| 19. *P. merrillii* | PMS0703-1        | 707        |                          | EU035310      |
| 20. *P. niger*    | CBS 213.48        | 611        |                          | AY558631      |
| 21. *P. noxius*   | Taiwan PN72.1     | 613-L      |                          | JQ005239      |
| *P. noxius*       | Taiwan PN1.2      | 609-M      |                          | JN836344      |
| *P. noxius*       | Taiwan PN29.1     | 609-M      |                          | JN836346      |
| *P. noxius*       | Taiwan PNA4.1     | 609-M      |                          | JN836346      |
| *P. noxius*       | Taiwan PNS.2      | 608-M      |                          | JQ003233      |
| *P. noxius*       | Taiwan PN4.2      | 607-M      |                          | JQ029276      |
| *P. noxius*       | Taiwan PN22.1     | 601-S      |                          | EF065630      |
| *P. noxius*       | CBS170.32         | 601-S      |                          | EF065631      |
| *P. noxius*       | Taiwan TF666      | 601-S      |                          | JQ003238      |
| *P. noxius*       | Malasia FRIM638   | 610-M      |                          | HQ400698      |
| *P. noxius*       | Malaysia FRIM618   | 602-S      |                          | HQ400699      |
| *P. noxius*       | Malaysia FRIM613   | 602-S      |                          | HQ400700      |
| *P. noxius*       | Malaysia FRIM551   | 603-S      |                          | HQ400702      |
| *P. noxius*       | Malaysia FRIM154   | 601-S      |                          | HQ400703      |
| *P. noxius*       | Malaysia FRIM147   | 599-S      |                          | HQ400704      |
| *P. noxius*       | –                 | 608-M      |                          | AB639002      |
| 22. *P. occidentalis* | CBS 196.55       | 706        |                          | AY558634      |
| 23. *P. pachyphloeus* = *Inonotus pachyphloeus* | CBS 193.37   | 571        |                          | AY558635      |
| 24. *P. pini*     | ATCC12240         | 635        |                          | AF250930      |
| 25. *P. pini* var. *cancriformans* | IMSNU 32031 | 636        |                          | AF200242      |
| 26. *P. pomaceus* | CBS 616.89        | 658        |                          | AF340765      |
| 27. *P. populus*  | CBS 498.76        | 636        |                          | AY558638      |
| 28. *P. punctatus* | CBS 282.77        | 714        |                          | AY558642      |
| 29. *P. repandus* | CBS 167.29        | 663        |                          | AY558640      |
| 30. *P. rhabarbarinus* | CBS 162.30       | 499        |                          | AY558640      |
| 31. *P. ribis f. ulicis* | CB5 579.50     | 653        |                          | AY558644      |
| 32. *P. rimosus* | MDJCBS586         | 608        |                          | DQ103885      |
Table 1 continued

| Species | Geographic origin | Strain no. | ITS sequence length (bp) | Accession no. |
|---------|-------------------|------------|--------------------------|---------------|
| 33. *P. robustus* | KCTC 6657 | 679 | AY558645 |
| 34. *P. senex* | CBS 442.76 | 578 | AY558647 |
| 35. *P. spiculosus* | KTCC 6658 | 641 | AY558648 |
| 36. *P. tremulae* | CBS 123.40 | 595 | AY558650 |
| 37. *P. tropicalis* | CBS 123.40 | 595 | AY558650 |
| 38. *P. tuberculosus* | CBS 617.89 | 636 | AF534077 |
| 39. *P. weirii* | CNU 6017 | 620 | AF251438 |

*a* The country where *P. noxius* was isolated

*b* CBS Centraalbureau voor Schimmelcultures, NPMU National Programme Management Unit, KCTC Korean Collection for Type Cultures, ATCC American Type Culture Collection, CFMR Colegiul Fizicienilor Medicali din România, FRIM Forest Research Institute Malaysia, IMSNU Institute of Microbiology, Seoul National University, CNU Collection of Newcastle University

*c* L: ITS type L; M: ITS type M; S: ITS type S

Table 2 List of hosts, locations, ITS information, GenBank accession no. of strains of *Phellinus noxius* from Taiwan used in the study

| Scientific name (common name) | Isolate | Location | Year of isolation | GenBank accession no. | Sequence ITS1/5.8S/ITS2 (bp) | ITS type |
|-------------------------------|---------|----------|------------------|----------------------|-------------------------------|----------|
| 1. *Annona squamosa* (custard apple) | PNA4.1 | Taitung County | 1996 | JN836346 | 609 | M6 |
| 2. *Araucaria cunninghamii* (hook pine) | PN29.1 | Taichung City | 2004 | JN836344 | 609 | M4 |
| A. cunninghamii | PN30.1 | Taichung City | 2004 | 609 | M4 |
| 3. *Averrhoa carambola* (carambola) | PNS1.1 | Tainan City | 1992 | 607 | M32 |
| 4. *Bauhinia × hybrid* (butterfly tree) | PN40.2 | Hualien County | 2005 | JQ003235 | 606 | M33 |
| 5. *Bauhinia variegata* (orchid tree) | PN7.1 | Nantou County | 1996 | 609 | M6 |
| 6. *Calocedrus formosana* (Taiwan incense cedar) | PN35.1 | Hualien County | 2005 | JN836349 | 609 | M9 |
| 7. *Casuarina equisetifolia* (ironwood tree) | PN35.2 | Hualien County | 2005 | 609 | M9 |
| 8. *Cinnamomum kotoensis* (botel tobago cinnamon tree) | PN74.2 | Taitung County | 2009 | 608 | M24 |
| C. kotoensis | PN74.1 | Taitung County | 2009 | JQ029271 | 608 | M27 |
| 9. *C. osmophloeum* (Taiwan cinnamon) | PN50.1 | Nantou County | 2006 | 601 | S1 |
| 10. *Cinnamomum camphora* (camphor) | PN51.1 | Nantou County | 2006 | 608 | M24 |
| C. camphora | PN32.1 | Taichung City | 2005 | 608 | M24 |
| C. camphora | PN32.2 | Taichung City | 2005 | 608 | M24 |
| 11. *Citrus limon* (lemon) | PN1.1 | Tainan City | 2003 | 609 | M1 |
| C. limon | PN1.2 | Tainan City | 2003 | 609 | M1 |
| C. limon | PN4.1 | Chiayi County | 2006 | 607 | M32 |
| Scientific name (common name) | Isolate | Location | Year of isolation | GenBank accession no. | Sequence ITS1/5.8S/ITS2 (bp) | ITS type |
|---|---|---|---|---|---|---|
| 12. Delonix regia (flame tree) | PN37.1 | Hualian County | 2005 | | 601 | S1 |
| D. regia | PN37.2 | Hualian County | 2005 | JQ029275 | 607 | M31 |
| D. regia | PN42.1 | Hualian County | 2005 | | 601 | S1 |
| 13. Dimocarpus longan (longan) | PNLn5.1 | Tainan City | 1992 | JQ003236 | 601 | S1 |
| D. longan | PNLn9.2 | Changhua County | 1998 | JQ003226 | 608 | M17 |
| D. longan | PNLn10.1 | Tainan City | 2003 | JQ003222 | 609 | M6 |
| D. longan | PNLn10.2 | Tainan City | 2003 | JQ003222 | 609 | M13 |
| D. longan | PNLn14.2 | Changhua County | 2006 | | 601 | S1 |
| 14. Diospyros kaki (persimmon) | PNPe1.1 | Chiayi County | 1991 | | 609 | M1 |
| 15. Duranta repens (creeping sky flower) | PN3.1 | Nantou County | 1996 | JQ003231 | 608 | M22 |
| 16. Enodotrya japonica (loquat) | PNLo3.1 | Taitung County | 1997 | | 601 | S1 |
| 17. Eucalyptus citriodora (lemon gum eucalyptus) | PN6.1 | Nantou County | 1996 | | 608 | M22 |
| 18. Ficus microcarpa (small-leafed banyan) | PN21.1 | Miaoli County | 2003 | | 608 | M24 |
| F. microcarpa | PN21.2 | Miaoli County | 2003 | | 608 | M24 |
| F. microcarpa | PN12.1 | Taichung City | 1996 | JQ029274 | 607 | M30 |
| F. microcarpa | PN26 | Nantou County | 2003 | | 608 | M24 |
| F. microcarpa | PN28.2 | Taichung City | 2004 | | 608 | M19 |
| F. microcarpa | PN49.2 | Taichung City | 2005 | JQ003227 | 608 | M18 |
| F. microcarpa | PN57.1 | Taichung City | 2005 | | 609 | M7 |
| F. microcarpa | PN75.1 | Taichung City | 2009 | | 607 | M30 |
| F. microcarpa | PN76.1 | Taichung City | 2009 | | 609 | M4 |
| 19. Ficus pumila var. awkeotsang (jellyfig) | PN10.1 | Chiayi County | 1991 | JQ029272 | 608 | M28 |
| 20. F. religiosa (botree fig) | PN90.1 | Taichung | 2009 | JN836342 | 609 | M2 |
| 21. Juniperus chinensis var. kaizuka (dragon juniper) | PN65.1 | Nantou County | 2007 | | 609 | M4 |
| 22. Kigelia pinnata (sausage tree) | PN14.1 | Nantou County | 1998 | JN836348 | 609 | M8 |
| 23. Koelreuteria henryi (flame gold-rain tree) | PN94.1 | Taichung City | 2009 | JQ003237 | 601 | S2 |
| K. henryi | PN33.2 | Hualian County | 2005 | JQ003223 | 609 | M14 |
| K. henryi | PN41.1 | Hualian County | 2005 | | 609 | M14 |
| K. henryi | PN41.2 | Hualian County | 2005 | | 609 | M14 |
| 24. Liquidambar formosana (maple) | R9218 | New Taipei city | 1992 | | 608 | M15 |
| 25. Litchi chinensis (litchi) | PN2.1 | Chiayi County | 1992 | JN836347 | 609 | M7 |
| L. chinensis | PN2.2 | Chiayi County | 1992 | | 601 | S1 |
| L. chinensis | PN5.1 | Kaohsiung City | 2003 | JQ029273 | 607 | M29 |
| L. chinensis | PN5.2 | Kaohsiung City | 2003 | | 607 | M29 |
| 26. Mangifera indica (mango) | PN4.1 | Changhua County | 2009 | | 609 | M6 |
| 27. Melaleuca bracteata 'Revolution Gold' (white cloud tree) | PN73.2 | Taichung City | 2009 | | 609 | M6 |
| Scientific name (common name) | Isolate | Location | Year of isolation | GenBank accession no. | Sequence ITS1/ITS2 (bp) | ITS type |
|-------------------------------|---------|----------|-------------------|-----------------------|-------------------------|----------|
| *M. bracteata* ‘Revolution Gold’ | PN73.1 | Taichung City | 2009 | 609 | M6 |
| 28. *Murraya paniculata* (orange jasmine) | PN5.1 | Nantou County | 1996 | 608 | M24 |
| *M. paniculata* | PN5.2 | Nantou County | 1996 | JQ003233 | 608 | M24 |
| *M. paniculata* | PN25.1 | New Taipei City | 2004 | 601 | S1 |
| *M. paniculata* | PN25.2 | New Taipei City | 2004 | 601 | S1 |
| 29. *Oncidium* Gower Ramsey | PN44 | Yungling County | 2005 | 601 | S1 |
| 30. *Osmanthus fragrans* (osmanthus) | PN140.1 | Changhua County | 2009 | JQ003221 | 609 | M12 |
| 31. *Psidium guajava* (guava) | PN98007 | Koahsiung City | 2009 | JQ029270 | 608 | M26 |
| 32. *Podocarpus macrophyllus* (long-leaved podocarpus) | PN98.3 | Taichung City | 2009 | 601 | S1 |
| 33. *Prunus armeniaca* (apricot) | PN72.1 | Taichung City | 2009 | JQ003239 | 613 | L1 |
| *P. armeniaca* | PN72.2 | Taichung City | 2009 | 613 | L1 |
| 34. *Prunus campanulata* (Taiwan cherry) | PN71.1 | Taichung City | 2009 | JN386350 | 609 | M10 |
| *P. campanulata* | PN71.2 | Taichung City | 2009 | 609 | M10 |
| 35. *Prunus mume* (plum) | PN12.1 | Koahsiung City | 1991 | JN836341 | 609 | M1 |
| *P. mume* | PN12.2 | Koahsiung City | 1991 | JN836345 | 609 | M5 |
| 36. *Prunus persica* (peach) | PN109.1 | Nantou County | 1996 | JQ003228 | 608 | M19 |
| *P. persica* | PN10.1 | Changhua County | 2005 | JQ003225 | 608 | M16 |
| 37. *Pterocarpus indicus* (rose wood) | PN104.1 | Taichung City | 2009 | JQ003220 | 609 | M11 |
| 38. *Pyrus pyrifolia* (pear) | PN4.1 | Miaoli County | 2003 | 601 | S1 |
| *P. pyrifolia* | PN4.2 | Taichung City | 1998 | JQ029276 | 607 | M32 |
| *P. pyrifolia* | PN9.1 | Nantou County | 2004 | JN836343 | 609 | M3 |
| 39. *Schinus terebinthifolius* (Brazilian peppertree) | PN48.1 | Taichung City | 2005 | 601 | S1 |
| *S. terebinthifolius* | PN48.2 | Taichung City | 2005 | 601 | S1 |
| 40. *Spathodea campanulata* (African tulip tree) | PN147 | Changhua County | 2009 | 601 | S1 |
| 41. *Sterculia nobilis* (ping-pong) | PN17.1 | Nantou County | 1999 | JQ003224 | 608 | M15 |
| *S. nobilis* | PN84.1 | Taichung City | 2009 | 607 | M32 |
| *S. nobilis* | PN124.1 | Taichung City | 2009 | 609 | M6 |
| 42. *Syzygium samarangense* (wax apple) | PNW1.1 | Chiayi County | 1991 | JQ003234 | 608 | M25 |
| 43. *Terminalia catappa* (Indian almond) | PN2.1 | Chiayi County | 1996 | 609 | M4 |
| *T. catappa* | PN63.1 | Changhua County | 2007 | 607 | M32 |
| 45. *Toona sinensis, Cedrela sinensis* (Chinese cedar) | PN64.1 | Taichung City | 2007 | JQ003230 | 608 | M21 |
| 46. *Vitis vinifera* (grape) | PNG1.1 | Nantou County | 1999 | 609 | M6 |
| *Zizyphus mauritiana* (Indian jujube) | PNZ2.1 | Koahsiung City | 2001 | JQ003229 | 608 | M20 |
as V1 to V20 in ITS1 and ITS2 but not 5.8S in the 91 Taiwanese isolates of P. noxius obtained in this study (Table 3). The variation included insertion, deletion and single nucleotide polymorphism. Some isolates showed single nucleotide polymorphism among chromosomes in the same isolate.

**Grouping based on ITS sequence length**

The examination of ITS nucleotide variation also revealed the possible division into three distinct groups based on sequence length among the 91 isolates of P. noxius from Taiwan (Table 4). Isolates with long sequence of 613 bp were termed type L. Only two isolates belonged to this type. Isolates with median sequence length of 606–609 bp were termed type M. The majority of the Taiwanese isolates with a total of 72 isolates belonged to this type. Type M was further divided into 33 subtypes based on single nucleotide polymorphisms, single nucleotide deletion (V12), double nucleotide deletion (V19) and single nucleotide insertion (V20) (Tables 3, 4). Isolates with short sequence of 601 bp were termed type S. Type S was further divided into two subtypes as a result of a single nucleotide polymorphism at position 114. Seventeen isolates belonged to this type.

Isolates of P. noxius from GenBank fitted or nearly fitted the M or S ITS types (in Taiwan; Table 1). Isolate CBS170.32 of unknown origin belonged to type S, so was the Japanese isolate. The isolate from India belonged to type M. Among the six isolates from Malaysia, isolate FRIM154 fitted the type S and isolate FRIM 638 nearly fitted the type M with 1 bp more than the Taiwanese type M. Isolates FRIM 618, FRIM 613 and FRIM 551 nearly fitted type S with 1–2 bp more than the Taiwanese type S, while isolates FRIM 147 was also close to type S with 2 bp less than the Taiwanese type S. However no isolates from other countries were founded to fit the Taiwanese type L in this study.

**Relation between ITS types and hosts and locations from where P. noxius was found in Taiwan**

Type L was detected only in Taichung City (Fig. 1). Type M was found in three cities and seven counties, while type S was found in two cities and eight counties. P. noxius was not found in Yilan County, Taoyuan County, Hsinchu Tounty and Pingtung County during this study.

Subtype S1 was found on 12 plant species located in three cities and seven counties, while subtype S2 was found only on flame gold-rain tree in Taichung City (Table 2). Other isolates found on flame gold-rain tree in

### Table 3 Nucleotide variation in ITS detected among isolates of Phellinus noxius in Taiwan

| Kind of variant | Sequence position | Nucleotide variation |
|-----------------|-------------------|----------------------|
| **ITS1**        |                   |                      |
| V1/V1*          | 19                | G, A/R               |
| V2              | 31                | T, C                 |
| V3/V3*          | 32                | G, C/S               |
| V4              | 114               | G, C                 |
| V5              | 116–117           | GGAGAG               |
| V6              | 117–118           | TG, AT               |
| V7              | 125–126           | TC, AT               |
| V8/V8*          | 129               | T, A/C               |
| V9              | 135–142           | ATTTATTC             |
| V10             | 152               | A, G                 |
| V11             | 168               | C, T                 |
| V12             | 193               | A                    |
| V13             | 197               | T, C                 |
| **ITS2**        |                   |                      |
| V14/V14*        | 420               | T, C/Y               |
| V15/V15*        | 442               | G, A/R               |
| V16/V16*        | 469               | A, G/R               |
| V17/V17*        | 546               | A, G/R               |
| V18/V18*        | 593               | C, G/S               |
| V19             | 594–595           | AC                   |
| V20             | 600–601           | C                    |

*The variants with an asterisk symbol represent isolates with single nucleotide polymorphisms among chromosomes in the same isolate.*
Hualian County belonged to subtype M14. This study also revealed that isolates of *P. noxius* obtained from the same plant species in the same location may belong to different subtypes. In Taichung City, isolates of *P. noxius* found on small-leafed banyan consisted of subtypes M4, M7, M18, M19 and M30. Similarly, isolates obtained from longan in Tainan City contained subtypes S1, M6 and M13. It was also found that isolates obtained from the same host in different locations may belong to the same subtype. For examples, subtype S1 on longan was found in Tainan City and Changhua County, while subtype M24 on small-leaved banyan was found in Miaoli County and Nantou County. Isolates obtained from different hosts in different locations may also belong to the same subtype. For examples, subtype M1 was found on lemon in Tainan City and on persimmon in Chiayi County, while subtype M6 was found

| **Table 4** ITS types and subtypes among *Phellinus noxius* isolates from Taiwan |
|---------------------------------|-------------------------------------------------|-------------------|-----------------|
| **ITS type and subtype** | **ITS sequence length (bp)** | **Nucleotide variation** | **Representative isolate (total no.)** |
| **Type L** | | | |
| L1 | 613 | V1, V3, V5, V15, V16, V19 | PN72.1 (2) |
| **Type M** | | | |
| M1 | 609 | | PNP1.2 (5) |
| M2 | 609 | V16 | PN90.1 (1) |
| M3 | 609 | V17 | PNP9.1 (1) |
| M4 | 609 | V18 | PN29.1 (5) |
| M5 | 609 | V1, V3 | PNP2.1 (1) |
| M6 | 609 | V14, V15 | PNA4.1 (8) |
| M7 | 609 | V15, V16 | PNL2.1 (2) |
| M8 | 609 | V3, V14, V15 | PN14.1 (1) |
| M9 | 609 | V1, V3, V16, V17 | PN35.1 (2) |
| M10 | 609 | V1, V3, V11, V15, V17 | PN71.1 (2) |
| M11 | 609 | V15*, V18* | PN104.1 (1) |
| M12 | 609 | V1, V15*, V16 | PN140.1 (1) |
| M13 | 609 | V14*, V15, V17* | PNLn102.1 (1) |
| M14 | 609 | V1*, V3*, V15*, V16* | PN33.2 (3) |
| M15 | 608 | V3, V12 | PN17.1 (2) |
| M16 | 608 | V3, V12, V17 | PNP10.1 (1) |
| M17 | 608 | V10, V12, V15, V16 | PNLn9.1 (1) |
| M18 | 608 | V1, V3, V11, V12 | PN14.2 (1) |
| M19 | 608 | V1, V3, V8, V11, V12, V16 | PN14.1 (2) |
| M20 | 608 | V1, V3, V6, V11, V12, V15 | PNZ2.1 (1) |
| M21 | 608 | V1, V2, V3, V11, V12, V15, V16 | PNL4.1 (1) |
| M22 | 608 | V1, V2, V3, V8, V11, V12, V13, V16 | PN3.1 (2) |
| M23 | 608 | V1, V2, V3, V6, V7, V11, V12, V15, V16 | PN70.2 (1) |
| M24 | 608 | V15, V16, V19, V20 | PNS.2 (9) |
| M25 | 608 | V1, V3, V15, V16, V19, V20 | PNP1.1 (1) |
| M26 | 608 | V3, V12, V15*, V16* | PN98007 (1) |
| M27 | 608 | V15, V16*, V19, V20 | PN74.1 (1) |
| M28 | 608 | V1, V3*, V15, V19, V20 | PNP10.1 (1) |
| M29 | 607 | V19 | PNL5.1 (2) |
| M30 | 607 | V16, V19 | PN12.1 (2) |
| M31 | 607 | V15, V16, V19 | PNP3.2 (1) |
| M32 | 607 | V1, V3, V15, V16, V19 | PNP4.2 (7) |
| M33 | 606 | V1, V2, V3, V6, V7, V11, V12, V15, V16, V19 | PNP40.2 (1) |
| **Type S** | | | |
| S1 | 601 | V3 | PNLn5.1 (16) |
| S2 | 601 | V4, V9 | PNP9.1 (1) |

*The variants with an asterisk symbol represent isolates with single nucleotide polymorphisms among chromosomes in the same isolate.*
Phylogenetic analysis based on ITS sequences among *Phellinus* species

The ITS sequences of 58 isolates belonging to 39 species of *Phellinus* retrieved from GenBank and seven *P. noxius* isolates representing type L, type M and type S of ITS sequences from Taiwan were used in the analysis of the phylogenetic relationship among *Phellinus* species. The result showed that all the isolates of *P. noxius* including isolates from Taiwan and other countries were in the same clade with 100% bootstrap support (Fig. 2). The sequence similarity between *P. noxius* and other *Phellinus* species was less than 85%. The species most closely related to *P. noxius* was *P. pachphloeus* with 83% similarity, whereas the most distant species was *P. badius* with only 67% similarity.

Discussion

Results from this study showed that the isolates of *P. noxius* from Taiwan can be divided into type L, type M and type S based on ITS sequence length. From 1991 to 2009, 2 type L isolates, 82 type M isolates and 17 type S isolates were found on 46 plant species in Taiwan (Table 2). To our best knowledge, this is the first report of division of isolate from the same fungal species into different groups based on ITS length. *P. noxius* was reported from Taiwan as early as 1928 (Sawada 1928). It is conceivable that type M and type S may have existed in Taiwan for a very long period of time and that type M may have evolved in Taiwan earlier and became the predominant type. Only two isolates of type L was obtained from apricot at Taichung City. It is possible that type L may be a recent mutation from subtype M25 through an 6 bp insertion at position 116–117 (V5), and deletion at position 600–601 (V20) (Tables 3, 4). However, the possibility that it may be due to host specificity of type L has not been ruled out.

The results also suggested the possibility that type S may originate from type M through an 8 bp deletion at the position between 135 and 142 (V9) (Tables 3, 4). After the deletion, the ITS sequences seem to become stable because there were only two subtypes among 17 isolates of type S obtained in this study. Moreover, the difference between subtype S1 and subtype S2 was the occurrence of a single nucleotide polymorphism at sequence position 114 (V4) in the latter.

*Phellinus noxius* is one of the plant pathogens with a very wide host range. Among the more than 200 plant species representing 59 families listed as hosts of *P. noxius* in the world, about half of them were reported for the first time from Taiwan (Ann et al. 2002). This is compatible with the discovery of great nucleotide variation in ITS region among isolates of *P. noxius* found in Taiwan in this study. The variation included 15 kinds of single nucleotide polymorphisms, three kinds of deletions and two kinds of insertions (Table 3).

Analysis of the ITS sequences of the Taiwanese isolates of *P. noxius* revealed that the 5.8 S region was identical in all isolates, while significant sequence variation was observed in ITS regions. This is in agreement with those reported with powdery mildews (Hirata and Takamatsu 1996) and *Fusarium* species (Naqvi et al. 2013). Our studies showed that the ITS1 was more variable than ITS 2 (Table 3). The former contained 10 single nucleotide polymorphisms, one 8 bp deletion, one 1 bp deletion and one 6 bp insertion, while the latter consisted of only five single nucleotide polymorphisms, one 2 bp deletion and one 1 bp insertion.

Phylogenetic analysis of ITS sequences among *Phellinus* species showed that isolates of *P. noxius* were in the same clade distinctly separated from other *Phellinus* species (Fig. 2). Phylogenetic relationship among *Phellinus* species based on ITS sequences has been reported previously (Shin 2001; Wagner and Fischer 2002; Jeong et al. 2005; Decock et al. 2006). However, none of them
Fig. 2 The (unrooted) distance tree of phylogenetic relationship of 17 Phellinus noxius isolates and other 38 species in Phellinus spp. based on the internal transcribed spacer (ITS1-5.8Sr DNA-ITS2) region of nuclear ribosomal DNA. Branch lengths and bootstrap (1000 replicates) were displayed in the distance tree by using neighbor-joining and bootstrap methods of Philip 3.67 software. The distance bar is corresponded to 10 substitutions per 100 nucleotide sites.
has included *P. noxius* in their studies. *P. noxius* has been transferred to *Phellinidium noxium* (Corner) Bondartseva & S. Herrera in 1992 (Bondartseva et al. 1992). However, *Phellinidium noxium* was distinctly separated phylogenetically from other *Phellinidium* species (Dai 2010), indicating that more study is needed in the future.

During this study, *P. noxius* was not found in the counties of Yilan, Taoyuan, Hsinchu and Pingtung (Fig. 1). This does not mean that the fungus was not present in those areas because detection of *P. noxius* in those counties had been reported previously (Ann et al. 2002).

**Conclusion**

The 91 isolates of *Phellinus noxius* obtained from 46 plant species in Taiwan showed 20 different kinds of variation including single nucleotide polymorphisms, deletion, insertion in ITS1 and ITS2, but none in 5.8S. The Taiwanese isolates of *P. noxius* were divisible into long (type L), median (type M) and short (type S) groups based on ITS sequence length. Phylogenetic analysis of ITS sequence among *Phellinus* species showed the isolate of *P. noxius* were in the same clade distinctly separated from other *Phellinus* species.

**Authors’ contributions**

WHK designed the experiments and wrote the manuscript; PJA, RFL and WHH conceived the experiments; JNT performed the experiments. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

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