

# Mating compatibility and phylogeography in *Pleurotus tuberregium*

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Genetic relationships were investigated among several populations of *Pleurotus tuberregium* from Nigeria, Papua New Guinea and New Caledonia. Intra-stock mating compatibility studies using progeny from two collections demonstrated a tetrapolar mating system for *P. tuberregium*. Inter-stock matings among the geographically distinct populations were compatible. All isolates were found to be intersterile with tester strains of other *Pleurotus* species, showing that *P. tuberregium* represents a unique intersterility group in *Pleurotus*. Nucleotide sequences of the ITS region of the rDNA gene were determined for 30 isolates and used to infer phylogenetic structure of populations. Phylogenetic analysis shows that African and Australasian-Pacific isolates form at least two distinct evolutionary lineages. Higher genetic divergence was observed among ITS sequences from the Australasian-Pacific region than among African isolates, which suggests a possible origin of *P. tuberregium* in the Australasian-Pacific region.

## INTRODUCTION

*Pleurotus tuberregium* (syn. *Lentinus tuberregium*) is the only species of *Pleurotus* known to produce fruit bodies from a globose true sclerotium. The sclerotia of *P. tuberregium* are often found in the field and may also develop during cultivation on lignocellulosic substrates. Sclerotia may grow to considerable size (up to 25 cm diam), are very dense and quite resistant to long periods of desiccation, and have been shown to support successive fruiting over consecutive seasons. Both sclerotia and fruit bodies of *P. tuberregium* are used for food and medicinal purposes in tropical Africa (Oso 1977, Nwokolo 1987, Isikhuemhen & Okhuoya 1995, 1996, Okhuoya, Eboigbe & Isikhuemhen 1996, Okhuoya, Isikhuemhen & Evue 1998).

The taxonomic position of *P. tuberregium* has been problematic. Because it produces leathery fruit bodies and possesses a dimittic hyphal system with intercalary skeletal hyphae, many previous authorities placed it in either *Panus* or *Lentinus* (Corner 1981, Pegler 1983). More recently, *P. tuberregium* was demonstrated to produce nematotoxic microdroplets in culture (Hibbett & Thorne 1994), supporting its classification in *Pleurotus* by Singer (1986). This placement is supported by molecular systematic studies that also place *P. tuberregium* with *Pleurotus* (Hitoshi & Takao 1995, Vilgalys *et al.* 1996).

The geographic distribution of *P. tuberregium* includes most

of equatorial Africa, India, Sri Lanka, southeast Asia and north Australia, as well as the southern Pacific (Pegler 1983). Because these populations have non-overlapping distributions, their study offers an opportunity for examining patterns of mating compatibility and phylogeography in a biogeographical context using molecular systematics. Molecular studies of ribosomal DNA sequence evolution have been very useful for understanding patterns of phylogenetic diversification in many fungi (Hibbett *et al.* 1995, O'Donnell *et al.* 1997) and especially *Pleurotus* (Vilgalys & Sun 1994, Vilgalys *et al.* 1996). In this study we address two aspects of genetic variation in *P. tuberregium* based on studies of: (1) mating compatibility among collections from Africa and Australasia; and (2) phylogeographic variation of ribosomal DNA genes of diverse geographic origin.

## MATERIALS AND METHODS

### Isolates

Isolates for this study originate from field collections and cultures representing different geographic regions in west Africa and the Australasia Pacific region (Table 1). Sources of isolates included spore prints taken in the field, as well as tissue and polyspore isolates obtained from field collections or from strains which were later fruited in the laboratory. Single basidiospore isolates were obtained from nine collections. Inter-specific mating compatibility was tested by pairing selected monokaryotic strains of *P. tuberregium* with tester strains of other *Pleurotus* species representing intersterility groups (IG) I–XV as reported in Vilgalys & Sun (1994) and

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**Table 1.** Collection data with GenBank accession numbers for isolates of *P. tuberregium*.

	Origin	Collection data	GenBank accession
Pt1	Benin region, Nigeria	Tissue isolate from sclerotium, August 1994	AF109983
Pt1.2–14		Single-basidiospore isolates from fruit body arising from sclerotial isolate Pt1	
Pt2	Benin region, Nigeria	Polyspore isolate from fruit body arising from sclerotium, collected August 1994	AF109981
Pt3	Fasidi region, Nigeria	Tissue isolate from sclerotium, collected August 1994	AF109982
Pt4	Owan region, Nigeria	Tissue isolate from sclerotium, collected August 1994	AF109985
Pt6	Oyo States region, Nigeria	Tissue isolate from a sclerotium, collected August 1995	AF109992, AF109996
Pt7	Oyo States region, Nigeria	Tissue isolate from sclerotium, coll. R. Arnold, August 1995	AF109993, AF109997
Pt8	Nigeria	—	AF109986
Pt9	Nigeria	—	AF109987
PtW1	Nigeria	—	AF109979
PtW4	Ebelle Territory, Edo State, Nigeria	Tissue isolate from sclerotium, coll. O. Isikhuemhen, October 1997	AF109980
PtW6	Benin City, Nigeria	Tissue isolate from sclerotium, June 1991	AF109984
PtWAT = WAT 22907	Mundenba S.W. Province, Cameroon	Tissue isolate from sclerotium, coll. R. Watling, April 1990	AF109988
PTV-2	Ghana	Tissue isolate, April 1995	AF109978
PTR-1	Ghana	Tissue isolate, April 1995	AF109989
PTR-3	Ghana	Tissue isolate, April 1995	AF109977
PTR-5	Ghana	Tissue isolate, April 1995	AF109976
DSH 92/155.4, 13	Madang Province, Papua New Guinea	Single-basidiospore isolates, coll. D. Hibbett, July 1992	AF109968, AF109967
RV95/174.15	Northern Territory, Australia	Single-basidiospore isolate, coll. R. Vilgalys, June 1995	AF109964
RV95/175.1	Northern Territory, Australia	Single-basidiospore isolate, coll. R. Vilgalys, June 1995	AF109965
RV95/945	Morobe Province, Papua New Guinea	Polyspore isolate, coll. R. Vilgalys, July 1995	AF109969
RV95/946.8	Morobe Province, Papua New Guinea	Single-basidiospore isolate, coll. R. Vilgalys, July 1995	AF109990, AF109994
RV95/947.1, 2, 7, 14	Morobe Province, Papua New Guinea	Single-basidiospore isolates, coll. R. Vilgalys, July 1995	AF109966
RV95/948.1	Morobe Province, Papua New Guinea	Single-basidiospore isolate, coll. R. Vilgalys, July 1995	AF109991, AF109995
RV95/949.2, 4, 6, 11, 16	Morobe Province, Papua New Guinea	Single-basidiospore isolates, coll. R. Vilgalys, July 1995	AF109970
RV95/950.2, 7, 10, 21, 22	Morobe Province, Papua New Guinea	Single-basidiospore isolates, coll. R. Vilgalys, July 1995	AF109971
Neda S467 (= TFM-M-S467)	Indonesia	Tissue isolate from sclerotium, H. Neda 1997	AF109975
Pt5 (= TFM-M-D779)	New Caledonia	Tissue isolate from sclerotium, coll. Y. Doi and H. Neda	AF109972
Pt5.1, Pt5.2		Monospore isolates derived from Pt5	AF109973, AF109974

Vilgalys *et al.* (1996). Tester strains were obtained from the Duke culture collection include: *P. ostreatus* D2195 (IG I), *P. pulmonarius* D2081 (IG II), *P. populinus* D1899 (IG III), *P. cornucopiae* D383 (IG IV), *P. djamor* D2014 (IG V), *P. eryngii* D2176 (IG VI), *P. cystidiosus* D417 (IG VII), *P. levis* D2120 (IG VIII), *P. dryinus* D2176 (IG IX), *P. tuberregium* Pt1.1, 1.2, 1.7, 1.11 (IG X), *P. sp.* D2319 (IG XI), *P. abieticola* RHP 6554.17 (IG XII), *P. sp.* D2061 (IG XIII), *P. australis* D2332 (IG XIV), and *P. purpureoolivaceus* D2342 (IG XV).

### Mating studies

The mating compatibility systems of two collections (Pt1 and DSH 92/155) were determined through intrastock pairings among single-basidiospore isolates. Intercompatibility between different collections was determined by pairing single-basidiospore isolates from each collection in all pairwise

combinations and later scoring for presence of clamp connections (Eger 1978). Pairings were performed in 90 mm Petri dishes containing 20 ml of 2% MEA (malt extract agar) inoculated with agar plugs (3 mm diam.) from actively growing monokaryons. In each plate, the agar plugs of two monocultures to be tested were placed 15 mm apart and incubated at 30 °C until a well developed contact zone was established. Pairings were examined twice (typically after 14 and 28 d) in order to establish that nuclear migration had occurred and remained stable. Pairings were respectively scored positive if clamp-connections could be observed under light microscope at 100–400 × magnification.

### DNA isolation, ITS amplification and sequencing

Cultures were grown on 2% MEA or PDA for 1–2 wk. Aerial mycelia were harvested using a sterilised spatula, transferred

into Eppendorf tubes, freeze-dried, and ground into powder using a plastic pestle (Kontes). DNA was isolated from culture tissues as well as dried fruit bodies using the procedure of Lee & Taylor (1990), followed by PCR amplification of the ITS rDNA region using primers ITS1 and ITS4 (White *et al.* 1990) and 5.8 S and 5.8 SR (Vilgalys & Hester 1990). Amplified PCR products were checked on agarose gels, and purified for sequencing using microfiltration (Ultrafree MC filters, Millipore Corp.). Sequencing reactions employed fluorescent dye terminator chemistry and were run on ABI 373 or 377 Automated Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA) using standard manufacturer's protocols. Both strands from each PCR product were sequenced. Sequence contigs were assembled and edited using Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI). In order to gauge the possible extent of intra-genomic variation which might exist among ITS sequences within an isolate, sequences were also determined for different single-spore isolates arising from the same parent (for two collections, DSH92/155 and Pt5).

### Phylogenetic analysis

Phylogenetic analyses based on maximum parsimony were performed using the PAUP\* (vers. 4.0d64, Swofford 1998). Sequences were manually aligned using PAUP\*, with gaps introduced where necessary to optimise the alignment. Unambiguously positioned single-base gaps were treated as a fifth character state in the analyses. Heuristic searches were conducted with the following settings: random addition sequence (100 replicates) with tree-bisection-reconnection (TBR) branch-swapping, starting trees obtained via stepwise addition, MULPARS option in effect, steepest descent option not in effect, MAXTREES setting unlimited, and branches having minimum length zero were allowed to collapse to yield polytomies. In the absence of relevant outgroup, trees were rooted using the midpoint method in PAUP\*. Branch robustness was evaluated with 100 bootstrap (Felsenstein 1985) replicate searches with settings as described above.

## RESULTS AND DISCUSSION

### Mating compatibility studies

Two sets of intrastock crosses were performed using single basidiospore isolates from Papua New Guinea (DSH 92/155) and Nigeria (Pt1). The results of both sets of pairings were consistent with a tetrapolar mating system, with four mating types represented from each parental stock (ratio of mating types for DSH 92/155 was 4:8:3:1 for A1B1:A2B2:A1B2:A2B1, respectively; for Pt1 the ratios were 5:5:1:4). These results are consistent with an earlier report of tetrapolarity in *P. tuberregium* by Petersen *et al.* (1997) which examined single-basidiospore progeny of collection DSH 92/155. Mating systems of other *Pleurotus* spp. that have been tested have also been tetrapolar (Vilgalys & Sun 1994, Petersen 1995, Vilgalys *et al.*, 1996, Zervakis & Balis 1996, Petersen & Hughes 1997, Petersen, Nicholl & Hughes 1997). In tetrapolar basidiomycetes, 25% of random

**Table 2.** Inter-isolate crosses of monokaryons of *P. tuberregium*.

	Pt5.1	Pt5.2	RV95/947.1	RV95/947.2	RV95/947.7	RV95/947.14	RV95/949.2	RV95/949.4	RV95/949.6	RV95/949.11	RV95/949.16	RV95/950.7	RV95/950.10	RV95/950.21	RV95/950.22
Pt1.2	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+
Pt1.5	+	+	+	-	-	+	+	+	+	-	+	-	-	+	+
Pt1.8	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+
Pt1.9	+	+	-	+	+	-	+	-	-	+	-	-	+	-	-
Pt1.10	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+
Pt1.11	+	+	+	-	+	+	+	-	+	+	+	-	+	+	-
Pt1.12	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+
Pt1.13	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-
Pt1.14	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-
Pt5.1	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Pt5.2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RV95/947.1															
RV95/947.2						+	-	+	+	+	+	-	+	+	+
RV95/947.7							-	-	+	+	-	-	+	+	-
RV95/947.14								-	-	+	-	-	+	+	-
RV95/949.2									-	-	+	-	-	-	+
RV95/949.4										+	-	-	-	+	+
RV95/949.6											-	-	+	+	+
RV95/949.11												-	-	+	+
RV95/949.16													+	+	+
RV95/950.7														-	-
RV95/950.10															+
RV95/950.21															-
RV95/950.22															+

+ Clamp connections observed (cross compatible).

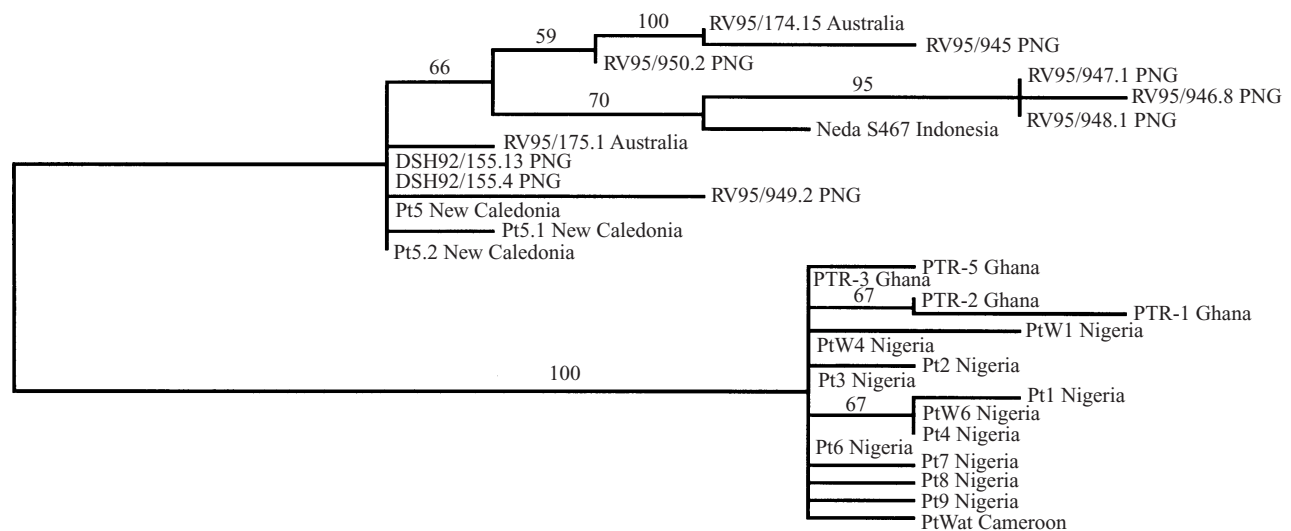
- Clamp connections not observed (cross incompatible).

**Table 3.** Mating frequency among different interstock pairings between geographic populations of *P. tuberregium* from Nigeria (NIG), New Caledonia (NCL), and Papua New Guinea (PNG).

	Number of crosses	Compatibility (%)
Intercollection crosses		
NIG/PNG	135	71
NIG/NCL	18	83
NCL/PNG	26	84
PNG/PNG	56	55
Intra-collection (Sib) crosses		
PNG/PNG	22	27

intrastock crosses are expected to be compatible (Carlile & Watkinson 1994). Although the number of intrastock crosses from our study is not very large, our results correspond well with theoretical expectation (Tables 2 and 3).

Interstock pairings among collections were largely inter-compatible. A sample cross matrix is shown in Table 2. By contrast, interstock crosses between strains of *P. tuberregium* with tester strains representing other intersterility groups in *Pleurotus* were always negative, indicating that *P. tuberregium* represents a single broadly distributed intersterility group, or biological species. Mating compatibility between collections from different geographic regions was generally high, with 70% or more of all interstock pairings being compatible (Table 3). A slightly lower frequency of compatible matings (55%) was observed for crosses made between single-spore isolates derived from collections made within a single locality in PNG (Table 3). We suspect that this lower frequency of mating



**Fig. 1.** Most parsimonious tree (1 of 6) resulting from phylogenetic analysis of ITS sequences from different isolates of *Pleurotus tuberregium*. Tree length = 41. Consistency index (CI) = 0.975. Values above branches show bootstrap support values based on 100 replications.

compatibility between strains is largely due to sharing of common mating factors between single-spore isolates, since some of these strains were collected from the same area (e.g. most of the collections from Papua New Guinea). Distinct patterns of shared A or B mating alleles were never obvious, however, even when many strains were paired in all possible combinations (e.g. Table 2).

During the course of this study, several isolates used for crossing studies died in culture or storage. We observed that cultures of *P. tuberregium* are best grown at 30–35°, and that most isolates die if stored at 4–6° even for short periods of time. Subsequent to making this observation, isolates were stored at only moderately low temperatures (15–20°) on sterilised hardwood sawdust substrate, with longer storage (years) possible on sawdust supplemented with vegetable oil (10% w/w). Sclerotia stored at lower temperatures were found to survive for longer times than at room temperature.

### ITS phylogeny and phylogeography

ITS sequences were determined from 30 isolates. Because of the small number of insertions/deletions ('indels'), sequences were easily alignable by hand. No sequence variation was observed in the flanking 18S and 25S rDNA regions, or within in the 5.8S rRNA gene. The size of the aligned ITS 1 region varied from 236 to 237 bp and contains a single informative indel. The ITS2 region ranged from 202 to 205 bp, and also contained a single 3 bp indel that was coded in the data matrix as a single evolutionary event. After removal of positions at the extreme 5' and 3' ends with missing data for several taxa, 590 characters remained in the analyses: of these, 553 characters were constant, 17 variable characters were parsimony-uninformative, and 20 were parsimony-informative.

Because rDNA exists as a multi-gene family in basidiomycetes, individual dikaryotic and monokaryotic strains can sometimes possess more than a single rDNA variant (Hibbett 1992). Although concerted evolution usually acts to

'homogenise' intragenomic variation, sequence heterogeneity could sometimes confound phylogenetic studies if more than one divergent rDNA sequence is present (O'Donnell & Cigelnik 1997). We, therefore, sought to address whether sequences determined by direct PCR-sequencing from genomic DNA might show any evidence for heterogeneity among rDNA sequences. Two single-spore isolates from collection DSH92/155 had identical ITS sequences, while ITS sequences of two single spores isolates (Pt5.1 and Pt5.2) differed by either none or one nucleotide position from their parental dikaryon Pt5. Sequence heterogeneity was observed for several isolates (RV95/946.8, RV95/948.1, Pt2, Pt4, Pt6 and Pt7) whose ITS sequences were not clearly readable beyond a certain position within the ITS1 region that contained a single-base insertion (TTT → TTTTT). Sequences for these isolates were still determinable by sequencing up to the insertion point from both directions. Though intragenomic variation must exist on some level in *P. tuberregium*, these results suggest that ITS sequences within populations and individual isolates from nature are typically highly uniform.

Parsimony analysis of the ITS data set yielded a single tree-island of six equally parsimonious trees with a length of 41 steps (consistency index = 0.975). All trees differed insignificantly by small rearrangements of only a few taxa. One most parsimonious tree is shown in Fig. 1 along with bootstrap values for well-supported clades. Two major lineages of ITS sequences are strongly supported (with 100% bootstrap support): an African clade with isolates from Ghana, Nigeria and Cameroon, and a Australasian-Pacific clade representing isolates from Indonesia, Australia, Papua New Guinea and New Caledonia. ITS sequences of the African and Australasian-Pacific collections differed in 11–20 positions and were clearly separated by phylogenetic analysis (100% bootstrap support) suggesting that these regions have diverged *via* reduced gene flow.

Minimal sequence variation was evident for ITS sequenced within the African clade, which differ from each other by only 0 to 5 base substitutions. The lack of genetic differentiation

among African isolates suggests a rather homogeneous gene pool of *P. tuberregium* in the regions sampled. By contrast, sequence variation within the Australasian isolates is higher (up to 10 substitutions between ITS sequences), with ITS sequences grouped into at least several well-supported lineages (66 to 100% bootstrap support, Fig. 1); these lineages do not, however, correspond with any particular geographic pattern. For example, some ITS sequences from Papua New Guinea (PNG) are more similar to ITS sequences from New Caledonia and Australia than with neighbouring collections from PNG (Fig. 1). These results could suggest there might be more gene flow within the Australasia-Pacific region, and also suggest an origin of diversification for *P. tuberregium* is in the Australasia-Pacific region.

Results from these phylogenetic studies strongly associated geographic isolation with patterns of genetic divergence in *P. tuberregium*, which is also congruent with marked patterns of phylogenetic diversification among geographically distant populations in other *Pleurotus* species (Vilgalys & Sun 1994). To our knowledge, this is the first evidence of a phylogeographic 'break' in fungi associated with disjunction between Africa and Australasia based on molecular evidence. The evolutionary forces which cause phylogeographic structure in this case are unknown, but likely arise from genetic drift between populations that have been geographically (and genetically) isolated. Genetic separation of African and Australasian-Pacific populations of *P. tuberregium* may be a consequence of the separation between the land masses of Africa and Australasia-Pacific 150 Myr years ago (Keast 1981), or may have occurred more recently. Comparison of molecular sequence divergence among populations (using a molecular clock) might help to answer these questions. Since fossil-based calibrations do not yet exist for ITS sequence divergence, we compared the divergence in our data to that used by other systematists who have attempted to date radiations in other fungi using molecular clocks (Berbee & Taylor 1993). We estimate the number of mutations between African and Australasian-Pacific clades to be 11 steps (based on ACCTRAN character-state reconstructions using PAUP\* on the most-parsimonious tree in Fig. 1). If we assume a constant rate of ITS sequence evolution (i.e. a molecular clock) and a divergence time between Africa-Gondwana of 150 million years, then the rate of divergence of the ITS gene in *P. tuberregium* is estimated to be 1.67% substitutions 100 Myr<sup>-1</sup>. This estimate seems very low, since it is only 1.67 times faster than another rDNA gene (18 S rDNA, with 1% divergence 100 Myr<sup>-1</sup>, Berbee & Taylor 1993) which is known to evolve much more slowly than the ITS region. Continental drift by itself thus appears unlikely as the primary factor responsible for genetic divergence between African *vs* Australasian populations.

Though ITS data strongly support evidence for vicariance and disjunction on a continental scale across the paleotropics, our results probably represent an underestimate of phylogenetic diversity within *P. tuberregium*, which has also been reported for east and central Africa, Madagascar, India, Sri Lanka and the Solomon Islands (Pegler 1983). Material from these regions was not available for this study. The distribution of *P. tuberregium* appears to be restricted to only a few regions

in the paleotropics, and we are not aware of any report for *P. tuberregium* from southern Africa, south America or the neotropics. Although our results based on ITS sequencing are well supported, additional geographic sampling, as well as analysis of additional gene phylogenies are still likely to aid in better understanding of phylogeography of *P. tuberregium* and related taxa.

Morphological characters of field collections associated with isolates from this study correspond with the description given by Pegler (1983) for *P. tuberregium*, which is characterised by a panoid/lentinoid fruiting habit with a well developed stipe, presence of dimitic tissues, and the clear association with sclerotia. Interestingly, not all field collections examined for this study were always associated with sclerotia (e.g. RV95/174 and RV95/175 from northern Australia were each found fruiting on wood without sclerotia). Several dikaryotic cultures used in this study were grown on sterilized sawdust and all were able to produce sclerotia. Only African isolates, however, appear to uniformly form sclerotia prior to fruiting under laboratory conditions, since Australasian strains were sometimes found to fruit directly on agar media or sterilized wood substrates under laboratory conditions (Isikhuemen & Vilgalys, pers. obs.). Growth behaviour and lignolytic enzyme production have also been observed to differ between collections from Nigeria and Papua New Guinea. Hybrid dikaryons produced by mating isolates from different geographic regions appear to grow as well as native isolates, and in at least one instance (a hybrid between a PNG and NIG) produced higher ligninolytic enzyme activity than either respective wild parental isolates (Isikhuemen & Nerud, unpubl.). Additional analysis of intercontinental hybrids might also provide a better understanding of genetic variation between disjunct populations in *P. tuberregium*, and may also provide a useful basis for breeding and improvement of this species to obtain high quality cultivars for commercial cultivation and other biotechnological applications.

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