Penicillium pedernalense sp. nov., isolated from whiteleg shrimp heads waste compost

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Novel Penicillium-like strains were isolated during the characterization of the mycobiota community dynamics associated with shrimp waste composting. Phylogenetic analysis of the partial β-tubulin (BenA) gene and the ribosomal DNA internal transcribed spacer region (ITS1–5.8S–ITS2) sequences revealed that the novel strains were members of section Lanata- Divaricata and were closely related to Penicillium infrabuccalum DAO MC 250537T. On the basis of morphological and physiological characterization, and phylogenetic analysis, a novel Penicillium species, Penicillium pedernalense sp. nov., is proposed. The type strain is F01-11T (=CBS 140770T=CECT 20949T), which was isolated from whiteleg shrimp (Litopenaeus vannamei) heads waste compost in the Pedernales region (Manabi province, Ecuador).

Ecuador is one of the leading producers of cultured whiteleg shrimp (Litopenaeus vannamei) in the world. Most hatcheries take their seawater from Pacific Ocean beaches. The principal production areas are El Oro, Guayas, Manabí and Esmeraldas (Hirono & Leslie, 1992). During the industrial processing of shrimp, an important waste (composed of the exoskeleton and the cephalothorax) is produced. This waste contains valuable components such as chitin, protein and pigments. Chitin and its deacetylated derivative chitosan have numerous applications in different areas: foods, pharmacy, cosmetics, textiles, biotechnology and agriculture (Gortari & Hours, 2013). In agricultural applications, the composting of shrimp heads is used to convert these wastes into valuable biological materials.

Composting is an aerobic biodegradative process, during which organic waste is biologically degraded by microorganisms to a stable, hygienic humus-like material beneficial to soil properties and plant growth. Many factors such as moisture content, oxygen input, temperature, pH, C:N ratio and available nutrients affect the composting process and the end product. Furthermore, these parameters are strongly connected to the microbial population (Partanen et al., 2010). Indigenous species of micro-organisms from compost have been described in different compost types, bacteria, actinobacteria and fungi being the most important present and active during a typical composting process. The addition of compost increases physical and chemical soil fertility, mainly by improving aggregate stability and by the incorporation of organic matter and nutrients. However, compost is much more complex than a fertilizer and its most significant value is to provide a high diversity of micro-organisms, involved in the nutrient cycle and in plant pathogenic biocontrol processes. In this respect, chitinous waste-based composts have potential value for pest management and biological control of crop diseases. The presence of chitin and its oligomer derivatives in compost could confer suppressive properties against plant pathogens by inhibiting growth of pathogenic fungi, inducing plant defence mechanisms and/or stimulating antagonistic microbial communities (Kendra & Hadwiger, 1984).

In this study, the fungal community dynamics associated with composting shrimp waste was analysed. Conventional morphological taxonomic methods and routine PCR and sequencing of internal transcribed spacer (ITS) and the

Abbreviations: BI, Bayesian inference; CI, consistency index; ITS, internal transcribed spacer; ML, maximum-likelihood; MP, maximum-parsimony; pp, posterior probability; RCI, rescaled consistency index; RI, retention index; TL, tree length.

The Mycobank (http://www.mycobank.org) accession number is MB 817801. The GenBank/EMBL/DDBJ accession numbers for the partial β-tubulin (BenA) gene and the ITS sequence of Penicillium pedernalense sp. nov. F01-11T (=CBS 140770T=CECT 20949T) are KU255396 and KU255398, respectively.

Two supplementary figures are available with the online Supplementary Material.
partial β-tubulin gene were used for identification. A large number of isolates corresponding to 12 different genera were obtained. Three Penicillium isolates (F01-11, F01-62, F02-53) with identical ITS and β-tubulin sequences were detected. Phylogenetic analysis and morphological and physiological characterization indicated that these strains represented a distinct species of the section Lanata-Divaricata most closely related to the recently described Penicillium infrabuccalum (Visage et al., 2016). On the basis of phenotypic and phylogenetic analysis, a novel species of Penicillium, Penicillium pedernalense sp. nov. (F01-11=CBS 140770T=CECT 20949T), is proposed.

Samples were obtained from a series of composts generated at the Universidad Laica ‘Eloy Alfaro’ de Manabí (ULEAM), Pedernales (Manabí province), Ecuador. Composting was done in five containers of 1 m³, mixing different proportions of wood chips, cow dung and whiteleg shrimp heads (obtained from shrimp ponds in the area of Pedernales). Compost piles were mixed for 4 months with consideration of the evolution of the temperature of the piles. Samples were collected before each mixture (four samplings) at three points of the inner part of each compost type. All samples were stored in sterile plastic bags and kept cooled during transport to the laboratory where they were processed. Ten grams of each sample was suspended in 90 ml of sterilized buffered sodium chloride peptone solution (Sigma) and serially diluted. For colony isolation, 0.1 ml of the sample was plated on dichloran rose Bengal chloramphenicol agar (DRBC; Pronadisa) and incubated at 25°C for 7–10 days. Fungal colonies with visibly different morphological appearance were isolated from each sample and plated on Blakeslee’s malt extract agar (MEAbl; Oxoid) and Czapek yeast extract agar (CYA; Difco). All isolates that showed different morphological characteristics (colony diameter, colour, pigments, exudates, etc.) in each culture medium, were maintained on slants of MEA, preserved in 30% (w/v) glycerol at −80°C and sent to the Instituto Canario de Investigaciones Agrarias (ICIA, Tenerife, Spain) for further analysis.

To determine the identity of the Penicillium isolates, they were subjected to a taxonomic study. Macromorphological and micromorphological analyses were performed as described by Visage et al. (2014) on the following media: MEAbI, malt extract agar (MEA), Czapek agar, CYA, CYA supplemented with 5% NaCl (CYAS), yeast extract sucrose agar (YES), oatmeal agar (OA), creatine sucrose agar (CREA) and dichloran 18% glycerol agar (DG18). Additionally, the culture medium 25% glycerol nitrate agar (G25N) was also used according to Pitt & Hocking (2009). For MEAbI and MEA, malt extract and peptone from Oxoid were used, and for YES and CYA, yeast extract from Difco was used. All macroscopic analyses were performed in duplicate at 25°C in darkness and results were recorded after 7 days, except on OA (incubated for 16 weeks) and CYA (incubated at 4, 25, 30 and 37°C). The detection of indole metabolites was performed with Ehrlich reagent using the filter paper method (Lund, 1995). The microscope slides were made in lactic acid (60%) from colonies growing on MEA and the morphological characteristics were examined under a differential interference contrast (Nomarski) optical microscope. The strains were deposited in the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) and the Colección Española de Cultivos Tipo (CECT, Valencia, Spain) culture collections.

Total DNA was extracted using the UltraClean microbial DNA isolation kit (MoBio Laboratories) according to the manufacturer’s instructions. The partial β-tubulin gene was amplified and sequenced using primers Bt2a and Bt2b (Glass & Donaldson, 1995), while the ITS region was amplified and sequenced using primers ITS1 and ITS4 (White et al., 1990). PCR amplification was performed as described by Visage et al. (2014). Amplicons were sequenced on an ABI PRISM 310 genetic analyser using a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. Sequences were deposited in the GenBank database of NCBI. The sequence data assembly and editing were performed using ChromasPro version 1.5. Comparisons with sequences from GenBank were done using BLASTN (Altschul et al., 1997). For phylogenetic analysis, sequences were retrieved from either the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/) or the CBS collections database (http://www.cbs.knaw.nl). Multiple sequences (individual and combined BenA and ITS dataset) alignments were performed with CLUSTAL W using MEGA version 5 software (Tamura et al., 2011). Phylogenetic trees were reconstructed using maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian inference (BI) analysis. ML and MP analyses were performed using MEGA 5. The robustness of branches was assessed by bootstrap analysis of 1000 replicates. For ML analyses the best-fit nucleotide substitution model for each dataset was selected with MEGA 5, according to the Bayesian information criterion (BIC) values (Schwarz, 1978). In this context, the best models (lowest BIC scores) were Tamura three-parameter (T92) with a gamma distribution (+G) and invariable sites (+I) for the ITS, K2+G for β-tubulin and K2+G+I for the concatenated dataset. The starting tree for the analysis was generated using the bio-neighbour-joining (NJ/BioNJ) option and nearest-neighbour-interchange (NNI) was used for tree searching. MP analysis was performed using the heuristic search option with the tree bisection and reconnection (TBR) algorithm and 1000 random sequence addition. Descriptive tree statistics including tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RCI) were calculated. Gaps and missing data were eliminated from the dataset (complete deletion option). BI analysis was performed in MrBayes v. 3.2.1 (Ronquist & Huelsenbeck, 2003) with the best-fit nucleotide substitution model based on the Akaiki information criterion (AIC) value (Akaikesi, 1973). The general time reversible with gamma distributed (GTR+G) model was the best fit for β-tubulin, and the GTR+G+I model was the best fit for the ITS and the concatenated.
dataset, BI was generated with the Markov chain Monte Carlo (MCMC) methodology to calculate posterior probabilities (pp) of the phylogenetic trees. Each analysis was run until the average standard deviation of the split frequencies dropped below 0.01 (1.5 million generations for each dataset). The sample frequency was set at 100 and 25 % of trees were removed as burn-in.

Composting is a complex microbiological process wherein different types of micro-organisms biodegrade organic waste. The results of this study reveal high microbial diversity in manure composts, and provide molecular evidence to support the effect of compost shrimp addition on microbial diversity. A total of 350 isolates were obtained during characterization of the compost dynamics of the fungal community. Aspergillus was the most commonly isolated genus throughout the composting process. Species corresponding to the sections Fumigati, Nidulantes and Versicolores were predominant. Aspergillus fumigatus and Aspergillus nidulans showed the highest isolation frequency and the greatest morphological diversity. Penicillium was the second most commonly isolated genus. Combined morphological and phylogenetical analysis showed that some of the Penicillium isolates belong to section Lanata-Divaricata. Among these isolates, we discovered three strains that did not fit into any known species. These strains (F01-11, F01-62 and F02-53) exhibited similar physiological and morphological characteristics and identical ITS and β-tubulin sequences, and represent the novel species described here.

Penicillium pedernalense sp. nov. showed spreading colonies on all examined agar media at 25 °C, with maximum growth on YES (colonies >50 mm diameter). It can be distinguished from other phylogenetically related species based on many morphological aspects. On CYA no growth at all, Penicillium mariae-crucis (on OA and MEA), Penicillium araracuarense (on OA and MEA), Penicillium simplicissimum (on OA and MEA) and Penicillium simplicissimum (occasionally on CYA and MEA) produce sclerotia.

The partial β-tubulin gene and the ITS region were analysed. Phylogenetic analysis conducted using the ML, MP and BI methods revealed the same tree topology, and consequently ML trees are used to present the results, with ML and MP bootstrap support and BI pp values marked on relevant branches. The β-tubulin gene dataset contained 523 characters including gaps, of which 201 characters were parsimony informative (TL: 543; CI: 0.491713; RI: 0.696703; RCI: 0.342578). The phylogram showed that Penicillium pedernalense sp. nov. formed a strongly supported subclade with the recently described Penicillium infrabuccalum DAOMC 250537, isolated from infrabuccal pockets of Carpenter ants (Camponotus pennsylvanicus) in New Brunswick, Canada (Visagie et al., 2016) (Fig. 1). The topology of the tree was coincident with the topology previously published by Visagie et al. (2015) in a detailed analysis of the section Lanata-Divaricata, and during the description of seven novel species of this section (Visagie et al., 2016). Comparison of the β-tubulin sequence of Penicillium pedernalense sp. nov. with other Penicillium type strains from the GenBank database showed 98.1% similarity (7 substitutions + 2 gaps) with Penicillium infrabuccalum (accession no. KT887817), 92.9% similarity (31 substitutions + 4 gaps) with Penicillium echinulonalgiovense (GU981631), 92.1% similarity (36 substitutions + 3 gaps) with Penicillium cata-tractum (KT887808), 91.1% similarity (39 substitutions + 5 gaps) with Penicillium panissanguineum (KT887823) and 90.4% similarity (42 substitutions + 6 gaps) with Penicillium mariae-crucis (GU981630). Phylogenetic analyses of the combined dataset sequences (partial β-tubulin gene and ITS) were performed. The combined dataset contained 1085 characters, of which 246 were parsimony informative (TL: 711; CI: 0.483826; RI: 0.680870; RCI: 0.329422). The topology of the tree was similar to that obtained with partial β-tubulin gene sequences (Fig. S1, available in the online Supplementary Material). ITS-based phylogenies confirmed the placement of Penicillium pedernalense sp. nov. in section Lanata-Divaricata. The ITS dataset contained 562 characters, of which 52 were parsimony informative (TL: 152; CI: 0.519737; RI: 0.725564; RCI: 0.377102). However, although the proposed novel species can be distinguished from most of the other species of the section, the ITS sequences showed 100% similarity with Penicillium infrabuccalum (KT887856) (Fig. S2). Likewise, Penicillium pedernalense sp. nov. strains showed 98.9% ITS sequence similarity (4 substitutions + 2 gaps) with Penicillium mariae-crucis (GU981593), Penicillium simplicissimum (GU981588) and Penicillium panissanguineum (KT887862), 98.3% similarity (7 substitutions + 2 gaps) with Penicillium cata-tractum (KT887847) and 98.1% similarity (8 substitutions + 2 gaps) with Penicillium echinulonalgiovense (GU981587).

The isolation of Penicillium pedernalense sp. nov. strains from wood/shrimp waste compost probably indicates that the proposed species has a good capacity for adaptation to high lignocellulosic and chitinous substrates. Species
Fig. 1. ML phylogenetic tree of *Penicillium pedernalense* sp. nov. and related species of the section *Lanata-Divaricata* based on partial β-tubulin gene sequences. ML bootstrap (bs) support above 70 % (left), BI pp above 0.85 (middle) and MP bootstrap support above 70 % (right) are given on each node. Branches which are not present in BI or MP trees are marked with an open circle. Branch values under 70 % bs or 0.85 pp are marked with a dash (–). Asterisks (*) represent branches with bs=100 % or pp=1. GenBank accession numbers of each sequence are shown in parentheses. *Penicillium glabrum* CBS 125543 was used as an outgroup. Bar, 0.05 substitutions per site.
belonging to the genus *Penicillium* are well known producers of cellulolytic and xylanolytic enzymes, and some of them are specific to the section *Lanata-Divaricata*. Abdel-Sater & El Said (2001) isolated different *Penicillium* species from wheat and rice straw and sugarcane bagasse, which produced endoxylanases. One of these species was *Penicillium oxalicum*. Krogh et al. (2004) showed that *Penicillium brasiliatinum* produces cellulolytic enzymes. Milagres et al. (1993) showed the high endoxylanolytic activity of *Penicillium janthinellum*. Likewise, this species has been described for its ability to produce chitinases (Di Giambettista et al., 2001). In addition, another species belonging to the section *Lanata-Divaricata*, *Penicillium excelsium*, was described associated with the ecosystem of the Brazil nut tree (*Bertholletia excelsa*) in the equatorial area (Amazon rainforest in Para and Amazon States, Brazil) (Taniwaki et al., 2015).

The datasets of the partial β-tubulin gene and the combined dataset (β-tubulin and ITS) clearly separated all the species of the section *Lanata-Divaricata*. *Penicillium pedernalense* sp. nov. and the type strain of *Penicillium infrabuccalum* (KT887817) showed 98.1 % β-tubulin gene sequence similarity, indicating that the two strains represent distinct species. Nevertheless, no differences were observed in the ITS sequences between these species and between others included in the section. This problem has been described in different *Penicillium* species (belonging to different sections) and many other genera of ascomycetes. The ITS region is not sufficiently variable for distinguishing between all closely related species (Skouboe et al., 1999; Schoch et al., 2012). As a consequence of this limitation, Visagie et al. (2014) proposed the use of the β-tubulin (BenA) gene as the best option for the identification of different *Penicillium* species. Nonetheless, morphological and phenotypic identification is necessary in this case and the results also suggest that *Penicillium pedernalense* sp. nov. and *Penicillium infrabuccalum* are not the same species. As mentioned above, *Penicillium pedernalense* sp. nov. grows faster on OA than *Penicillium infrabuccalum*, and produces more strongly coloured colonies on YES and on the reverse of MEA. Nevertheless, the strong acid reaction and the good growth and sporulation on CREA are the most relevant differences between *Penicillium pedernalense* sp. nov. and phylogenetically related species. Acid production on CREA is also a consistent and stable characteristic and is often useful for distinguishing between closely related species (Frisvad, 1981; Visagie et al., 2014). An exception in the present study was *Penicillium cataractum* due to its capacity to form colonies 13–45 mm in diameter with an acid reaction; however, this species shows more restricted growth on MEA and OA than *Penicillium pedernalense* sp. nov. and produces clear exudates on CYA.

Based on phenotypic and phylogenetic analysis, strains F01-11T (=CBS 140770T=CECT 20949T), F01-62 and F02-53 (=CBS 140769=CECT 20950) isolated from whiteleg shrimp waste compost in the Pedernales region (Manabí province, Ecuador) represent a novel species of *Penicillium* in the subgenus *Aspergilloides* section *Lanata-Divaricata* [according to the classification of Houbraken & Samson (2011)], for which the name *Penicillium pedernalense* sp. nov. is proposed.

**Taxonomy**

**Latin diagnosis of Penicillium pedernalense**


*Penicillos biverticillatae interdum divaricatae, in superficie poratae; stipa parietibus laevis 90–500 × 2.5–3.5 mm; metulae 8.5–15.5 × 2.5–3.6 mm; phialidiae ampulloformes 6.5–9.0 × 2.4–3.2 µm; conidia globosa vel subglobosa, laevia vel subulatrix exasperata, 2.0–3.2 µm.*


**Description of Penicillium pedernalense**

*Penicillium pedernalense* (pe.der.nal.en’se. N.L. neut. adj.) named after Pedernales, the collection site of the holotype.

**Etymology.** Latin, *pedernalense* (pe.der.nal.en’se. N.L. neut. adj.) named after Pedernales, the collection site of the holotype.

**Typus.** Ecuador: Pedernales, province of Manabi (0.0728° N 80.0406° W; 33 m.a.s.l.), isolated from whiteleg shrimp heads (*Litopenaeus vannamei*) waste compost, Sep. 2013, collected by F. Laich, J. Andrade, isolated by F. Laich, J. Moreira, F. Arias. Holotype strain F01-11T. Ex-type culture was deposited in CBS (CBS 140770T) and CECT (CECT 20949T). Mycobank accession number: MB 817801. GenBank database accession numbers: BenA=KU255396, ITS=KU255398.

**Macroscopic characteristics.** On CYA at 25 °C after 7 days, colonies are 40–42 mm in diameter, radially sulcate, texture velutinous or subfloccose, mycelium dense and white (Fig. 2a). Conidial production moderate (more

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abundant at the centre of the colony), coloured dull green to bluish green. Exudate and soluble pigment absent. Reverse pale brown. At 30 °C, colonies 44–47 mm in diameter. No growth observed at 37 or 4 °C. On MEA and MEAb at 25 °C for 7 days, colonies 32–39 and 27–28 mm in diameter, respectively, with similar morphological characteristics; plane, velutinous, mycelium white visible at the margins only (1–2 mm wide). Conidial production dense over the entire colony area, bluish green becoming olive green with age. Exudate and soluble pigment absent. Reverse orange–brown with some small greenish areas. On YES agar at 25 °C for 7 days, colonies 54–55 mm in diameter, irregularly sulcate, protuberant or convolute, velutinous or subfloccose, mycelium dense white to greyish. Conidiogenesis moderately dense, in colours similar to those on CYA. Exudate and soluble pigment absent. Reverse strongly coloured, light orange–brown. On CREA at 25 °C for 7 days, colonies (33) 37–39 mm in diameter, plane, velutinous (similar that on MEA), good growth and strong acid production. On OA at 25 °C for 7 days, colonies 43–45 mm in diameter, radially sulcate, velvety or subfloccose, mycelium white, visible at the margins. Conidial production dense, olive green at the centre of the colony and bluish green at the margins. Exudate and soluble pigment absent. On DG18 agar at 25 °C for 7 days, colonies 28–30 mm in diameter, plane, velvety or subfloccose, low, mycelium white. Conidial production very light, white to pale orange. Exudate and soluble pigment absent. Reverse pale yellowish orange. On G25N agar at 25 °C for 7 days, colonies 13–14 mm in diameter, plane, floccose, coloured buff with light sporulation. Reverse pale yellow. On CYAS at 25 °C for 7 days, colonies 30–32 mm in diameter, radially sulcate, velutinous, mycelium white at the margins. Conidial production light, pale green. Exudate and soluble pigment absent. Reverse pale orange brown. Sclerotia absent under all culture conditions assayed.

**Microscopic characteristics.** Conidiophores borne from mycelium surface, stipes variable in size (90–500×2.5–3.5 µm), delicate and slender, usually with smooth or finely rugose walls. Penicilli predominantly terminal biverticillate but also with some divericulate branching. Metulae in verticils of 2–4, variable in size, 8.5–15.5×2.5–3.6 µm, each bearing...
3–6 phialides. Phialides ampulliform, 6.5–9.0 × 2.4–3.2 μm, narrowing abruptly to distinct neck. Conidia commonly globose to subglobose, 2.0–3.2 (2.6±0.3) μm, typically smooth or verruculose-walled. Conidial chains usually columnar, 50–300 μm in length (Fig. 2b–g).

**Ehrlich test.** No reaction.


**Diagnostic features.** *Penicillium pedernalense* sp. nov. is classified in section *Lanata-Dirivaricata*. The phylogenies based on the single *BnA* gene and the combined datasets (partial β-tubulin gene and the ITS region) resolved it in a well-supported clade with *Penicillium infrabucaulcum* (Figs 1 and S1). Likewise, *Penicillium pedernalense* sp. nov. forms colonies larger than 30 mm on CREA with good sporulation and strong acid production, while *Penicillium infrabucaulcum* grows weakly with no acid production. Moreover, *Penicillium pedernalense* sp. nov. grows faster on OA and produces more strongly coloured colonies on YES (dull green to bluish green) and on the reverse of MEA (orange–brown) than *Penicillium infrabucaulcum*.

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**References**


