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Abstract	Investigations on enzymes of industrial interest from Fungi were conducted in order to define new phylogenetic tools based on the DNA sequences encoding these enzymes. Microtests suitable for high throughput enzyme activity analysis were applied. PCR primers were designed to allow amplification of given target regions of genes and sequencing. These sequences were tested as phylogenetic tools and compared to phylogenetic tree constructed from already proven reliable molecular tools.
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Abbreviation key

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ITS	Internal Transcribed Spacer
RPB2	RNA Polymerase II subunit B2

1 Background and Objectives

Fungal taxonomy is a crucial basis in microbiology, and consequently in Microbial Resources Centres for typing new strains in laboratory culture conditions. The correct identification and classification are also essential in the biotechnological industry, where fungi are strong contenders. Improper identification may result in considerable costs and safety problems. In recent years, a more precise assessment of identification and phylogenetic relationships in Fungi has been achieved using molecular markers including the ribosomal sequences, and partial sequences of house-keeping genes coding for subunits of RNA polymerase, actin and β-tubulin. In addition, correlation between phylogenetic proximity of fungal strains and their enzymatic properties has been described: (i) *Aspergillus niger* aggregate isolates, clustered according to their ability to produce various types of feruloyl esterases, enzymes involved in the degradation process of the plant cell wall polymers [1] and (ii) Chinese strains of the white-rot fungus *Pycnoporus,* exhibited high level of laccase production, blue copper enzyme oxidizing a wide range of polyphenols and environmental pollutants [2]. Based on this last study, laccase encoding gene, *lac3-1,* was successfully used to infer the phylogeny of *Pycnoporus* species and could highlight enzyme functional diversity associated with biogeographical origin of the strains [3].

Within the framework of the sub-task JRA2.3.1, enzyme activities of industrial interest from Fungi were investigated in order to define new phylogenetic tools based on the DNA sequences encoding these enzymes. Relevant groups of filamentous fungi, provided by the European Microbial Collections of EMbaRC project and involved in plant cell wall degradation, i.e. white-rot basidiomycetes and the genus *Aspergillus,* were selected to illustrate this approach.

2 Fungal resources

2.1 White-rot basidiomycetes

Among the white-rot basidiomycetes, the order Polyporales is the most representative of saprophytic homobasidiomycetes causing wood decay, and its high lignolytic potential is known through the production of multicopper oxidoreductases of industrial interest, e.g. laccases. These enzymes of industrial interest were successfully used in the bioremediation of polycyclic aromatic hydrocarbons, pulp and paper applications including wheat straw pulp, cross-linking of agro-industrial polysaccharides into biopolymers and in an industrial process of flavonoid production (oligorutins with anti-oxidant, anti-inflammatory and anti-ageing activities), compatible with cosmetic and pharmaceutical formulation guidelines [4]. Based on literature [5], the expertise of Pr. Stalpers from CBS-KNAW and works carried out at INRA CIRM-CF, 91 strains of Polyporales were initially selected, i.e. 20 from CBS-KNAW, 12 from MUCL and 59 from INRA CIRM-

Champignons Filamenteux. Finally, only 50 strains, representing 27 species and belonging to the complex '*Trametes*-group' *sensu* Ryvarden [6] (i.e. exclusion of the genus *Trichaptum* and of *Trametes cervina*), and the closely related genera *Coriolopsis, Lenzites* and *Pycnoporus*, were selected. That included *Coriolopsis polyzona, Trametes betulina, T. cingulata, T. gibbosa, T. hirsuta, T. junipericola, T. lactinea, T. ljubarskyi, T. maxima, T. meyenii, T. ochracea, T. pubescens*, *T. socotrana, T. suaveolens, T. versicolor T. villosa, Lenzites elegans, L. menziesii, L. warnieri, Pycnoporus cinnabarinus, P. sanguineus, P. coccineus* and *P. puniceus. Daedaleopsis tricolor, Hexagonia itida, H. mimetes* and *Trametella trogii* were used as outgroups. Twenty-nine strains were isolated from fresh sporocarps collected in Europe, French Guiana and French West Indies (i.e. Martinique and Guadeloupe) between 2007 and 2010 and deposited at INRA CIRM-CF. The other strains were provided by CBS-KNAW, MUCL and INRA CIRM-CF culture collections. Largely described in Polyporales species and suitable for biotechnology applications, laccase activity was chosen to be followed among the strains of the complex *Trametes* group.

2.2 Genus Aspergillus section Nigri

The genus *Aspergillus* is largely related for its ability to produce polysaccharide degrading enzymes, such as cellulases and hemicellulases including feruloyl esterases, enzymes involved in the hydrolysis of the ester bonds between hydroxycinnamic acids and sugars present in hemicelluloses [7]. This interesting group of enzymes has a potentially broad range of applications in the pharmaceutical and agri-food industries. Feruloyl esteraes have the ability to not only degrade plant biomasses but also to release ferulic acid, suitable precursor of vanillin by microbial bioconversion [8]. In this study, the feruloyl esterase Type A activity, specific of methoxylated hydroxycinnamic acids, was targeted. In order to increase the diversity of the strains tested, especially considering feruloyl esterase type A activity, the study of the genus *Aspergillus,* initially limited to the group *A. niger* aggregate, was extended to *Aspergillus* section Nigri. Seventy-nine strains (with 24 type strains) representing 21 species were selected: *A. aculeatinus, A. aculeatus*, *A. awamori, A. brasiliensis, A. carbonarius, A. costaricaensis, A. ellipticus, A. foetidus, A. heteromorphus, A. homomorphus, A. ibericus, A. japonicus, A. lacticoffeatus, A. niger, A. phoenicis, A. piperus, A. sclerotiicarbonarius, A. sclerotioniger, A. tubingensis, A. uvarum, A. vadensis).* The strains were provided by CBS-KNAW, CABI, UMinho-MUM and INRA CIRM-CF.

3 Methods

3.1 Culture conditions and high throughput enzyme analysis

Microtests suitable for high throughput enzyme analysis were developed based on previous works [9]. 10-ml miniaturized fungal cultures were carried out in 16-well baffled deep microplates. Microcultures were inoculated with mycelial fragments for non-sporulating fungi (i.e. *Trametes*). Incubation of miniaturized cultures was performed at constant temperature under shaking (Infors HT Switzerland). The fungal cultures were grown in a maltose medium supplemented with 1 % ferulic acid as inducer for laccase production [10]. In the case of feruloyl esterase A production by the strains of *Aspergilli,* 1.5 % sugar beet pulp was added as inducer to fungal cultures inoculated with spores. Under these culture conditions, incubations should be carried out in 500 ml baffled flasks containing 100 ml medium [7]. Only enzyme assay could be robotically conducted.

Enzyme assay was performed using chromogenic compounds as substrates, i.e. ABTS for laccase activity [10], and methyl sinapinate (MSA) and 5-O-trans-feruloyl-l-arabinofuranose (FA), specific substrates of feruloyl esterase A [7,9]. The assays were adapted to 96-well plates and automated liquid handling robot system. Enzyme activities were automatically measured each day and the results were expressed in nkat/ml of culture medium. The robot used is a Tecan Genesis Evo 200 (Tecan Lyon, France) with a eight-needle pipetting arm, two microplate handling arms, a shaking unit, a Branson 200 ultrasonic cleaner (Branson Ultrasonic, Geneva Switzerland), a Tecan Infinite 200 microplate reader and a automated sealer (Agilent Technologies). The robot and the microplate reader are driven by GEMINI and MAGELLAN software (Tecan).

3.2 Genomic DNA isolation, PCR amplification, sequencing and phylogenetic analysis

Genomic DNA was isolated from mycelial powder (40-80 mg on a wet weight basis) using the kit Nucleopsin Plant II as previously described [2].

3.2.1 Proven loci as molecular markers

ITS1-5.8S rRNA gene-ITS2 and partial regions of RPB2 and β–tubulin genes were analyzed. The ITS region was amplified and sequenced using the ITS1/ITS4 primer sets [11]. The RPB2 region for *Trametes* group was amplified and sequenced using the primer set bRPB2-6F, bRPB2-7.1R [12]. β-tubulin fragments from *Aspergillus* strains were amplified and sequenced using the primer sets Bt2a and Bt2b as previously described [1]. The degenerate inosine(I)-containing primers

Bsens (5'-ATCAC(A/T)CACTCICTIGGTGGTGG-3') and Brev (5'-CATGAAGAA(A/G)TGIAGACGIGGG-3') were specifically designed for the *Trametes* group from alignment of β-tubulin genes from *Trametes versicolor*, *Polyporus lepideus*, *Schizophyllum commune*, *Coprinus cinereus*, and *Pleurotus sajor-caju* (NCBI accession numbers AY944859, AY944857, X63372, AB000116, AF132911, respectively) [3].

3.2.2 Genes encoding for targeted enzymes as molecular markers

Partial regions of laccase *lac3-1* gene from *Trametes* strains were amplified and sequenced using the primer sets F2-R8. The two degenerate primers F2 (5'-CA(C/T)TGGCA(C/T)GG(A/G)TTCTTCC-3') and R8 (5'-GAG(A/G)TGGAAGTC(A/G)ATGTG (G/A)C-3') were designed to match, respectively, the copper-binding domains I and IV, highly conserved in blue copper oxidases such as laccases [3]. The sequences of F2 and R8 were based on the alignment of the corresponding nucleotide regions of the basidiomycete laccases from *P. coccineus*, *P. sanguineus*, *Lentinula edodes*, *Coriolus hirsutus* and *Pleurotus sajor-caju* (NCBI accession numbers AB072703, AY458017, AB035409, AY081775, AJ507324, respectively). The PCR products were further cloned into the pGME®-T Easy vector (Promega), following the manufacturer's protocols [3].

Partial regions of *faeA* gene from *Aspergillus* section Nigri strains were amplified and sequenced by combination of various pairs of primers designed from the sequence of *Aspergillus tubingensis faeA* gene (NCBI accession number Y09331.2), i.e. non-degenerate primers FAEAS (5'-ATGAAGCAATTCTCTGCA AAA-3') and FAEAR (5'-GCCTCACAGCACTGGACCTCATCCCC AG-3') and degenerate primers FAEASD1bis (5'-ATGAAGCAATTCTCYGCA AAA-3'), FAEASD2 (5'-ACRCARGGCATCTCCGA-3'), FAEASD3 (5'-ATGGCCACY ATCTCCBAAG-3'), FAEASD4 (5'-CTC CGCGACGACASCAG-3'), FAEARD1bis (5'-GCCTCACAGCACTGGACYTCATCCCCAG-3'), FAEARD2 (5'-TCMACGCTCCAGTACTC-3') and FAEARD3 (5'-GCTGCGCGGYTCGCCGAA-3'). with R, S, M, Y, and B representing G/A, G/C, T/C and G/T/C, respectively.

3.2.3 Sequencing and phylogenetic analysis

The PCR products were sequenced by GATC Biotech AG (Konstanz, Germany) or Cogenics (Meylan, France). The nucleotide sequences (only exons for β-tubulin, RPB2, laccase, and feruloyl esterase A gene fragments) were aligned using Clustal W algorithm; the alignments were then hand-refined. Phylogenetic analyses were performed from single genes or from combined genes using Bayesian or Neighbour Joining Methods.

4 Results

4.1 Phylogeny of Trametes group using laccase lac3-1 gene

This approach was based on recent works carried out on *Pycnoporus*, a four species cosmopolitan polyporoid genus, known especially for producing high redox potential laccases suitable for white biotechnology. The functional *lac3-1* gene, dominant gene in this group, was targeted to infer the phylogenetic relationships within the genus *Pycnoporus*, in addition to already proven loci, rRNA and β–tubulin gene. These three genomic fragments, with respect to *Pycnoporus* species, could provide effective molecular tools for routine identification and comparison of strains in laboratory culture conditions. The laccase gene *lac3-1* could highlight enzyme functional diversity associated with biogeographical origin of the strains [3].

To apply this approach to the complex *Trametes* group, corresponding to the genus *Trametes* and related genera *Coriolopsis*, *Lenzites* and *Pycnoporus*, it was necessary to build a robust phylogeny based on already proven loci. A well-resolved phylogenetic reconstruction of the *Trametes* group based on the Bayesian analysis of concatenated sequences of ITS region and RPB2 gene was proposed and accepted recently for publication in *Fungal Diversity* (Welti et al., 2012, DOI 10.10007/s13225-011-0149-2). Congruent results were obtained from analyses of ribosomal LSU sequences downloaded from GenBank, whilst β-tubulin sequences, too similar, gave a very weak resolution. The existence of three main lineages in the *Trametes*-clade was revealed (cf. Annex_Fig.1): (i) a so far monospecific lineage represented by *Artolenzites elegans*; (ii) a lineage including the genus *Pycnoporus* in its traditional sense and several species usually classified in the genus *Trametes* (*T. cingulata*, *T. lactinea*, *T. ljubarskyi*, *T. menziesii*); (iii) a lineage corresponding to the core genus *Trametes*, including type species of *Coriolopsis* and *Lenzites*. Morphological features from the corresponding herbarium vouchers completed this study. A partial systematic arrangement of the *Trametes* clade is proposed, with the introduction of a new genus: *Leiotrametes* Welti & Courtec. *gen. nov*., and two new combinations: *Leiotrametes lactinea* (Berk.) Welti & Courtec. *comb. nov*. and *L. menziesii* (Berk.) Welti & Courtec. *comb. nov*..

Laccase activity was robotically measured from 7-day microcultures grown in maltose medium supplemented with ferulic acid. As shown in Annex_Fig.1, laccase activity was measured in all the clades, but at a higher level in the core genus *Trametes* and in the genus *Pycnoporus,* which confirmed laccase gene presence in most species. PCR amplification resulted in laccase F2-R8 products of about 1600 bp. Comparison between gene and predicted cDNA fragment sequence (laccase from *Trametes versicolor*: NCBI accession number N°CAA77015), showed the corresponding partial coding regions were interrupted by introns, whose number, length and position differed from a gene to another. While with the species of *Pycnoporus*, only one dominant

gene, *lac 3-1*, was cloned and could allow to analyze the phylogenetic relationships in this genus [3], numerous genes encoding different laccase isoenzymes were cloned from the same strain, whatever the species studied. At least six genes encoding laccase isoenzymes have been found within *Trametes* species, unfortunately all of them being amplified by our primer set F2/R8. Such functional diversity is of great interest on an industrial point of view; however it prevents the phylogenetic analysis using functional laccase gene within the complex *Trametes* group.

4.2 Phylogeny of Aspergillus section Nigri using feruloyl esterase A gene (faeA)

Previous works revealed the presence of gene *faeA* in genomic DNA of various species of *Aspergillus* [13]. In addition, it was shown that the *Aspergillus niger* aggregate isolates clustered according to their ability to produce various types of feruloyl esterases [1] and that activity profiles of the feruloyl esterases were able to correctly predict the taxonomic class and family from 34 Ascomycota [14].

Before to evaluate the gene *faeA* as molecular tool within the *Aspergillus* section Nigri, a robust phylogeny of the selected strains was built based on ITS and β-tubulin sequences, as already described [15,16]. For the 79 selected strains of *Aspergillus* section Nigri (provided by CBS-KNAW, CABI, UMinho-MUM and INRA CIRM-CF) including 24 type strains and corresponding to 21 species, well-resolved phylogenetic trees based on ITS region and partial region of β-tubulin gene were constructed as references. ITS data were produced during this study, while only a part of β-tubulin data were produced, the other part being retrieved from GenBank. Neighbour-joining phylogenetic tree inferred from ITS sequences clearly differentiated five clades: (i) *A. niger* aggregate, a biseriate clade, including *A. niger A. lacticoffeatus, A. awamori*, *A.costaricaensis, A. tubingensis, A. vadensis A. piperis, A. foetidus* and *A. brasiliensis* as a sub-clade, (ii) a biseriate clade characterized by wide spores including *A. carbonarius, A. sclerotioniger*, with *A. ibericus* and *A. sclerotiicarbonarius* as *sub-clades* (iii) an uniseriate clade with *A. aculeatinus, A. aculeatus, A. japonicus* and *A. uvarum,* (iv) a clade including uncommon species *A. heteromorphus* and *A. ellipticus,* and (v) a clade with unresolved s*pecies A. homomorphus* (cf. Annex_Fig.2).

Partial regions of *faeA* gene were amplified from genomic DNA of the 79 strains of *Aspergillus* section Nigri by combination of various pairs of primers designed from the sequence of *Aspergillus tubingensis faeA* gene (NCBI accession number Y09331.2). Only 42 PCR products could be obtained and sequenced. The 42 sequences of partial regions of *faeA* gene belonged to 42 strains (including 13 type strains), and corresponded to 17 species with representatives of the five major clades of section Nigri. Neighbour-joining phylogenetic tree inferred from the *faeA* gene sequences distributed the strains into three well-supported groups (cf. Annex_Fig.3): (i) a group corresponding to all the strains of *A. brasiliensis*, (ii) a group with exclusively strains of *A. niger*

aggregate and (iii) a mixed group including monoseriate and biseriate strains, even from species found in group 2. So, *faeA* tree was totally incongruent with species reference tree, inferred from ITS and β-tubulin data within *Aspergilli* section Nigri [15,16]. Consequently, *faeA* gene may not be accurately used as phylogenetic tool. A real functional diversity was however revealed within this group of *Aspergillus* for *faeA* gene, which could allow to search out feruloyl esterases A with novel properties of industrial interest. In parallel, feruloyl esterase activity was robotically measured for these strains using methyl sinapinate (MSA), and 5-O-trans-feruloyl-l-arabinofuranose (FA), specific substrates of feruloyl esterase A. The higher activities were distributed in all the clades inferred from *faeA*-tree, without specificity (cf. Annex_Fig.3).

Conclusion

Microtests suitable for high throughput enzyme activity analysis were developed for the targeted enzymes of selected fungal groups, i.e. laccase activity for *Trametes* group and feruloyl esterase A for *Aspergillus* section Nigri. The results obtained were correlated with genes encoding enzymes, whose fragments were successfully amplified and sequenced after efficient design of primers and cloning in the case of laccase. While within genus *Pycnoporus,* member of the *Trametes* group, *lac3-1* gene could provide an effective molecular tool for routine identification of *Pycnoporus* species and highlight enzyme functional diversity associated with biogeographical origin of the strains [5], laccase genes within the *Trametes* group and feruloyl esterase A gene within *Aspergilli* section Nigri, could not be used as phylogenetic tools. The existence of numerous laccase isoenzymes coded by gene families impossible to amplify specifically, and the incongruence of *faeA* gene tree with referenced trees constructed with proven molecular loci, prevented the use of these functional genes as phylogenetic tools. However, a huge functional diversity was revealed through this study, enlarging the panel of industrially useful enzymes.

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Annexes

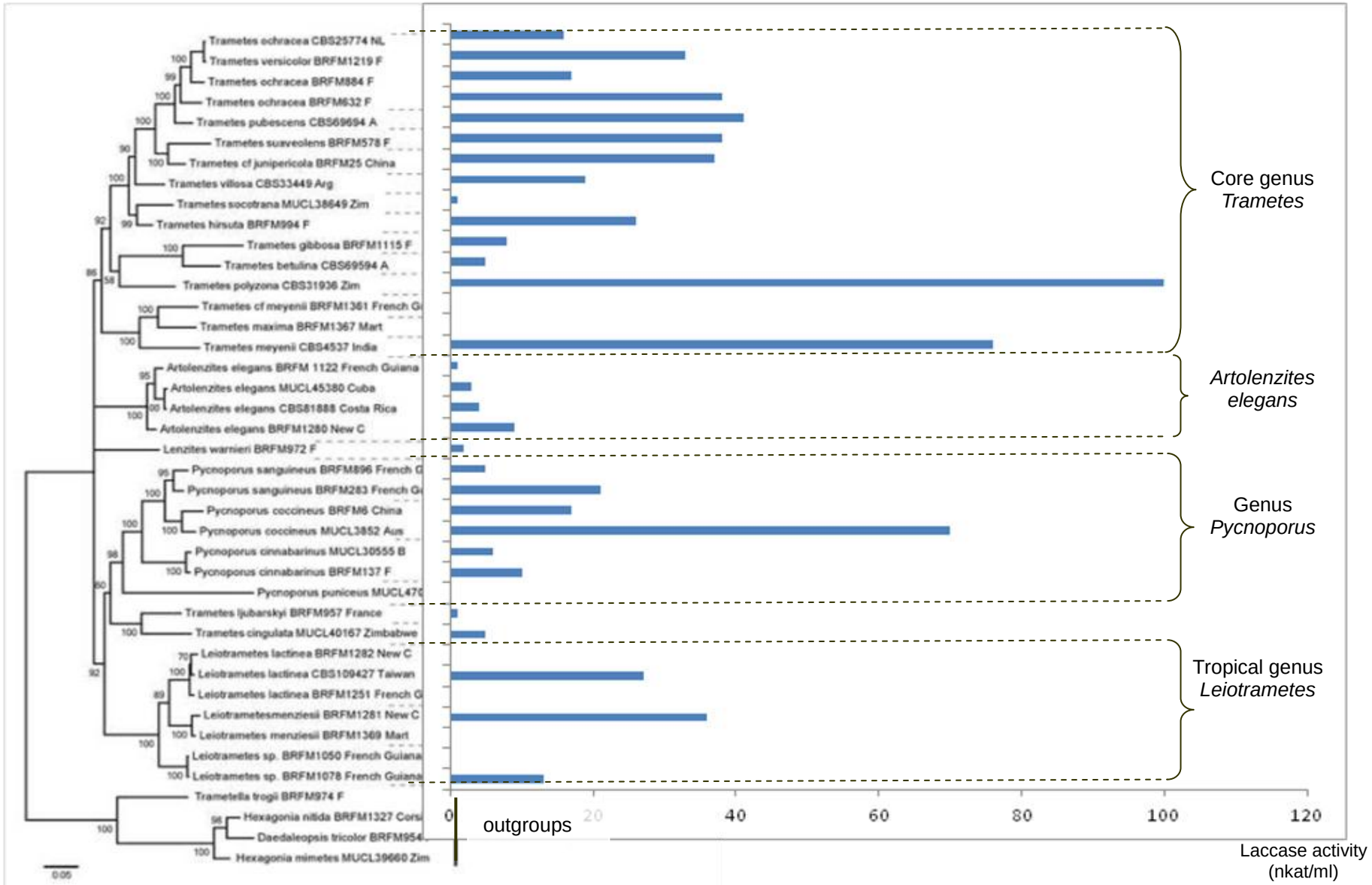


Fig.1 : Phylogenetic reconstruction of the *Trametes* group based on the combined analysis of ITS1-5.8S rRNA gene-ITS2 and RPB2 gene and laccase activity in microcultures grown in the presence of ferulic acid.

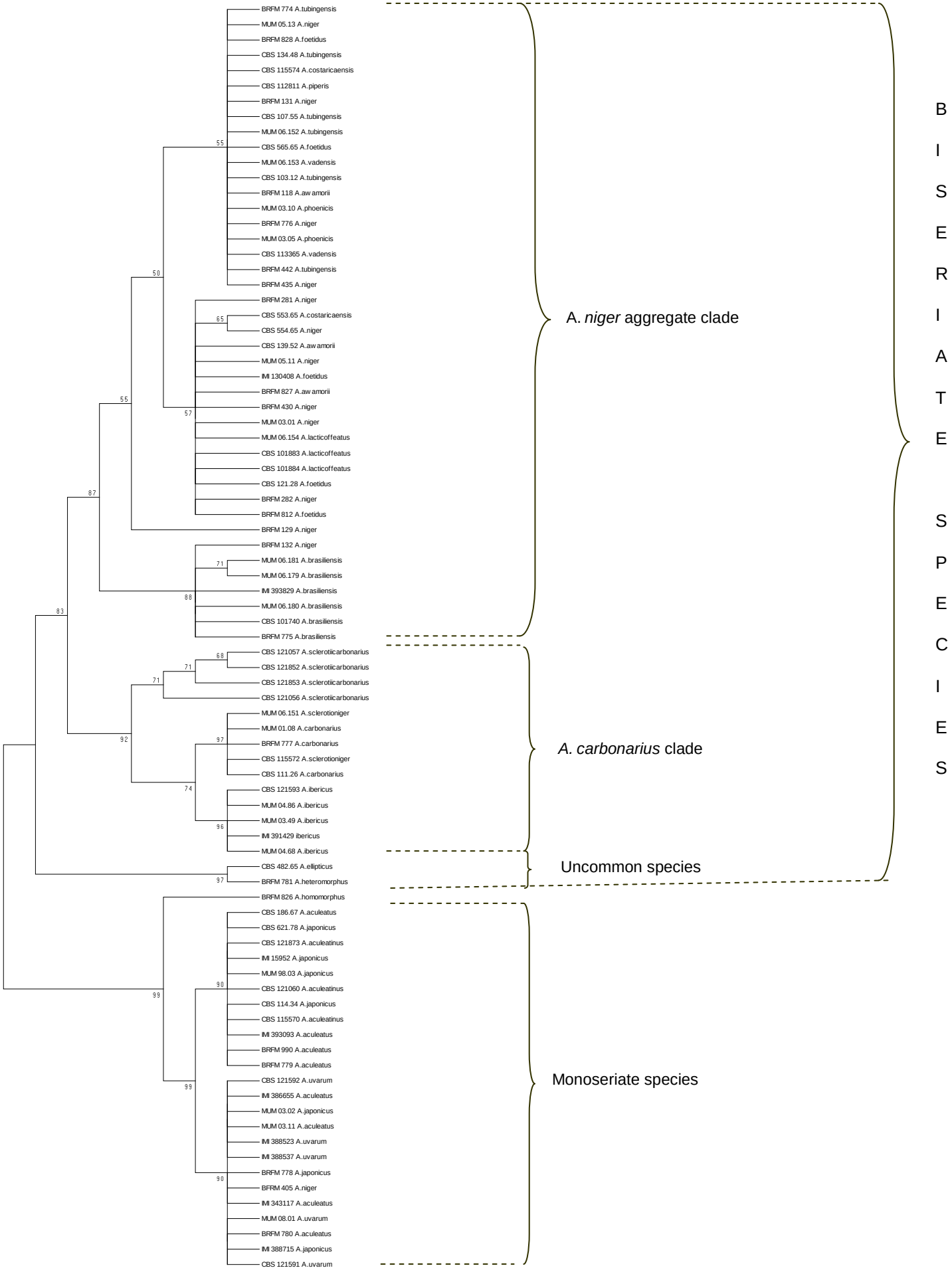
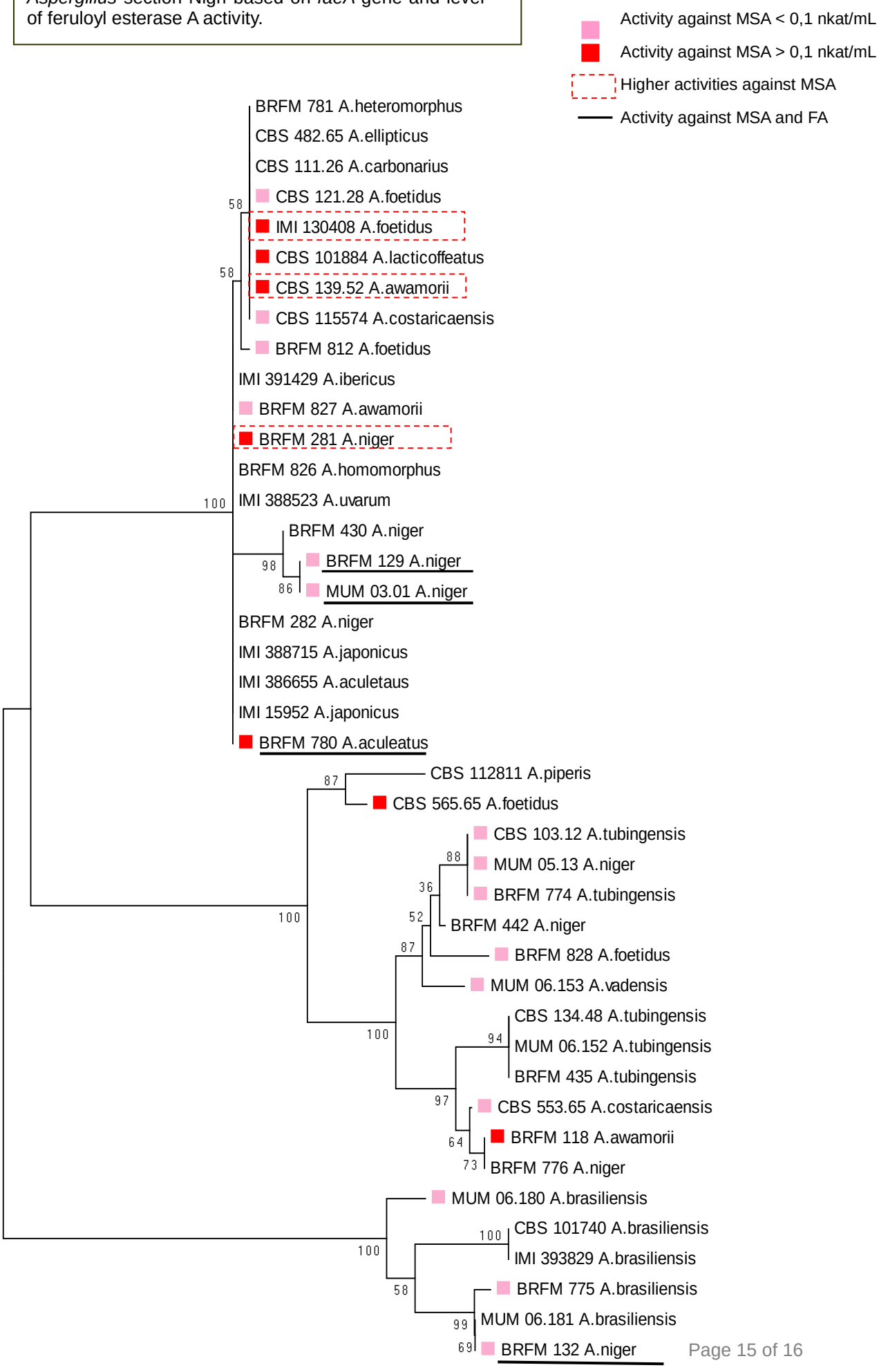


Fig. 2 : Reference tree of *Aspergillus* section Nigri based on ITS data

Fig. 3: Phylogenetic tree of the selected strains of *Aspergillus* section Nigri based on *faeA* gene and level of feruloyl esterase A activity.



Significance of this deliverable

Despite previous correlations between phylogenetic proximity of fungal strains and their enzymatic properties (like laccase encoding gene which was successfully used to infer *Pycnoporus* species phylogeny), the attempt described here to use genes of industrial enzymes as molecular tools in the identification within *Trametes* group and *Aspergillus* section Nigri was not successful.

However, regarding the enzymes of industrial interest explored, a large diversity was evidenced, with isoenzymes families shared by several species, and promising physico chemical properties that can now be explored for innovative applications.