

Aspergillus affinis sp. nov., a novel ochratoxin A-producing *Aspergillus* species (section *Circumdati*) isolated from decomposing leaves

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Two ochratoxin A (OTA)-producing *Aspergillus* isolates, recently collected from submerged riparian decomposing leaves in Italy, were found to have a similar morphology to *Aspergillus cretensis* (subgenus *Circumdati*, section *Circumdati*). However, marked differences emerged between these two novel isolates and *A. cretensis* as the former displayed different colony features and had larger vesicles, metulae, phialides and conidia, as well as a distinct sclerotial form and size. In order to determine the taxonomic status and to infer the evolutionary relationships of these two morphologically identical isolates, a molecular phylogenetic analysis was performed on all the officially recognized lineages in the section *Circumdati*. The DNA sequences and the deduced amino acid residues from the nuclear loci were analysed. Both rRNA and protein coding genes were assessed, which are widely used to differentiate taxa belonging to genus *Aspergillus* at various evolutionary levels. The 5.8S rDNA gene and internal transcribed spacers (ITS), the D1/D2 domains of the 28S rDNA gene, a region of the tubulin beta chain gene (*benA*) and part of the calmodulin gene (*cmd*) were amplified by PCR and then sequenced. The analysis of the rRNA regions and of the *benA* and *cmd* sequence data indicated that the two isogenic isolates belonged to a genetically distinct OTA-producing species of the genus *Aspergillus*. The isolates are proposed as representing a novel species, *Aspergillus affinis* sp. nov., with the type strain ATCC MYA-4773^T (=CBS 129190=417). Phylogenetically, *A. affinis* sp. nov. appeared to be very closely related to *A. cretensis*, from which it could be distinguished by means of a morphological trait analysis.

INTRODUCTION

A large number of studies have demonstrated that molecular approaches based on DNA sequencing combined with traditional methods based on phenotypic features are the most accurate means of reliably characterizing species of the genus *Aspergillus*. In general, the genus *Aspergillus*, which is

currently divided into seven subgenera, each of which is subdivided into sections, is analysed at the molecular inter-section level by means of the nuclear rRNA gene operon (often the ITS1-5.8S rDNA-ITS2 and the 28S rDNA gene, together known as the ID region; Peterson, 2008), and at the phylogenetic intra-section level by means of two nuclear protein coding genes, i.e. the tubulin beta chain gene (*benA*) and the calmodulin gene (*cmd*) (Geiser *et al.*, 2007; Samson *et al.*, 2007; Samson & Varga, 2009; Sørensen *et al.*, 2011, among others). The molecular taxonomy of the section *Circumdati* has recently been revised by Peterson (2008), whose phylogenetic analysis was based on the ID region and three protein encoding genes, including *benA* and *cmd*. Peterson largely confirmed the results of a previous single locus study in which seven novel species were described (Frisvad *et al.*, 2004), and identified an undescribed species. This section currently comprises the teleomorphic species

Abbreviations: ITS, internal transcribed spacers; MP, maximum-parsimony; NJ, neighbour-joining; OTA, ochratoxin A.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the ID region and the *benA* and *cmd* genes obtained in the present study are available in Table S1, available in IJSEM Online.

A supplementary table and two figures are available in the online version of this paper.

The Mycobank accession number for *Aspergillus affinis* sp. nov. is MB517245.

Neopetromyces muricatus and more than 20 recognized species of the genus *Aspergillus* that are, despite being closely related, phenotypically and phylogenetically diverse.

In this paper, two ochratoxin A (OTA)-producing *Aspergillus* isolates that morphologically resemble *Aspergillus cretensis* (section *Circumdati*), but could be distinguished from the latter on the basis of macro- and microscopical traits, were subjected to a molecular phylogenetic investigation. The strains were collected from a freshwater micro-fungal community related to plant litter decomposition. The aim of the study was to gain a higher taxonomic resolution than that provided by morphological studies and to gain insight into the evolutionary relationships of these two peculiar *Aspergillus* isolates. In order to address this issue, the morphological characteristics and DNA sequences (obtained by a multilocus approach) of the isolates, their ability to produce OTA, and their ecological distribution were compared with those of members of other known lineages within the section *Circumdati*.

METHODS

Strains. *Aspergillus* strains 417 and 418 were isolated from submerged decomposing leaf litter collected from two different sites in a river spring area (see below) in June 2009. In the laboratory, a 30 µl sample of gently homogenized damp leaf litter from each of the two sites was diluted (10^{-4}) and then spread on Sabouraud agar plates. Subsequently, some *Aspergillus* strains that resembled *A. ochraceus* were identified among the purified micro-fungal colonies and isolated for more detailed analyses.

The submerged vegetable litter examined in this study came from the Sacco River (a second-order stream) located in the south-east of the Lazio region (central Italy), where the alder (*Alnus glutinosa* L.) is the most common riparian forest species (also including *Salix alba* L., *Populus tremula* L. and *Populus nigra* L.). At the time of sampling, the water temperature ranged from 13 °C to 16 °C while the dissolved oxygen was 8–9 mg l⁻¹ (G. Galante, personal communication).

Media and growth conditions. According to standardized descriptions, isolates 417 and 418 were examined morphologically, cultured in Czapek yeast extract agar (CYA), Blakeslee's malt extract agar (MEA) and G25N agar, in 9 cm polystyrene Petri dishes, and incubated at 5 °C, 25 °C and 37 °C for 7 days in the dark, as described by Pitt (1979). The capitalized colour names and codes used to describe the colony colours refer to those described in Kornerup & Wanscher (1967).

Micromorphological features. The micromorphological structures (vesicles, phialides, metulae, conidia and sclerotia) were measured and photographed as described by Davolos *et al.* (2012).

OTA assay. *Aspergillus* strains 417 and 418 were grown at 25 °C for 10 days in potato dextrose agar (PDA) slants until sclerotia were evident. Aliquots (0.2 ml) from a suspension containing ca. 500 sclerotia ml⁻¹ were inoculated in 25 ml CY broth, and then incubated at 25 °C in the dark. Three samples were analysed after 3, 5 and 8 days of incubation. Each sample was filtered through Whatman grade no. 4 filter paper. The resulting mycelium was dried at 80 °C for 48 h and then weighted, while aliquots (5 ml) of the culture filtrate were acidified using 500 µl 0.1 M H₃PO₄ and extracted with ethyl acetate (v/v); the organic phase was then recovered. The extraction process was repeated three times. Extracts were pooled, dried at 35 °C under a nitrogen flow, redissolved in 300 µl water:acetonitrile

50:50 (v/v) acidified using 1 % formic acid, and then analysed by HPLC using an Agilent 1200 liquid chromatograph equipped with a spectrophotometer diode array detector. An Agilent YMC-Triart C18 column (3.0 µm, 150 × 2.1 mm ID) and a pre-column packed with the same stationary phase were used. The eluent was constituted by water:acetonitrile 50:50 (v/v) acidified by 1 % formic acid (flow rate of 0.2 ml min⁻¹). OTA was identified and quantified (limit=1 ng), according to the external standard method of integrating peak areas showing the retention time and the absorbance spectrum (200–450 nm) of the corresponding OTA standard (Sigma-Aldrich) (see Remiro *et al.*, 2010; Reverberi *et al.*, 2010). The standard calibration solution of OTA was prepared at a concentration of 1 ng µl⁻¹ by appropriately diluting the stock solution (500 µg ml⁻¹ in toluene acidified with 1 % formic acid). The calibration curve was conducted over a 2–30 ng range and yielded a correlation coefficient (R²) of 0.999.

DNA extraction, PCR amplification and DNA sequencing.

Genomic DNA was prepared from 3-day-old CYA cultures, according to the protocol based on enzymic digestion as detailed in Davolos *et al.* (2012). The final genomic DNA concentrations were 100–110 ng µl⁻¹ as determined by OD₂₆₀.

PCR amplification was performed using *Taq* DNA polymerase with aliquots of genomic DNA as the template. For the 5.8S rDNA gene, ITS1, ITS2 and for the D1 and D2 region of the 28S rDNA genes (together known as the ID region), the PCR-mediated reaction was performed using the primers cited in Davolos *et al.* (2012). The PCR amplification conditions were: 2 min at 95 °C, followed by 35 cycles, each consisting of 60 s at 95 °C, 50 s at 50 °C and 2 min at 72 °C; the final PCR extension step was 10 min at 72 °C. In addition, a region of ca. 570 bp of the tubulin beta chain (β -tubulin) gene (*benA*) was amplified by the primers Bt2as 5'-GGTAACCAAATCGGTGCTGCTT and Bt2bs 5'-ACCCTCAGTGTAGTGACCCTTG (present study), shortened versions of primers Bt2a and Bt2b (Glass & Donaldson, 1995). The genetic region of the β -tubulin monomer examined in this study encodes part of the N-terminal domain [includes residues in which two parallel β -strands (B2 and B3) alternate with an α -helix; see Nogales *et al.*, 1998]. The PCR cycling parameters were: 2 min at 95 °C, followed by 35 cycles, each consisting of 45 s at 95 °C, 50 s at 52 °C and 45 s at 72 °C; the final extension step was 10 min at 72 °C. Furthermore, a 690 bp-long region of the calmodulin gene (*cmd*), flanking the first and fourth exons, was amplified and sequenced using the primers cmdln-f: 5'-ACAGGTCTCCGAGTACAAGG (present study; designed according to the *cmd* sequences of *Aspergillus* species available from GenBank) and cmdA1 (Wang & Zhuang, 2007). The PCR amplification program for the *cmd* region was the same as that used for the *benA* section, the only differences being the initial denaturation temperature, the annealing time/temperature and the extension time, which were 3 min, 50 s at 51 °C and 50 s, respectively.

All the PCR products were verified and then sequenced as detailed in Davolos *et al.* (2012).

Gene sequence and phylogenetic analysis. The nucleotides and the inferred amino acid residues were compared with data available in the GenBank databases using the NCBI BLAST server. The sequence alignment was constructed using the CLUSTAL_X v.1.8 program. A phylogenetic analysis was conducted in MEGA 4 (Tamura *et al.*, 2007) using the neighbour-joining method (NJ), as specified by Davolos *et al.* (2012). Moreover, phylogenetic analyses were carried out in PAUP* 4.0b10 (Swofford, 2003) using maximum-parsimony (MP) with weighted codon positions (transversions were weighted five times more than transitions) and by calculating 1000 bootstrap replicates. Bayesian analyses were performed in MrBayes v.3 (Ronquist & Huelsenbeck, 2003), as fully reported in Davolos *et al.* (2012).

The partition homogeneity test implemented with PAUP* 4.0b10, using 1000 replicates and the heuristic general search option, revealed

concordance for the ID region, the *benA* and *cmd* datasets ($P=0.42$); a phylogenetic analysis in which the data from all these loci were pooled was also performed. The DNA sequences of the ID region and of the *benA* and *cmd* genes obtained in the present study have been deposited in the GenBank (NCBI) database (see Table S1, available in IJSEM Online).

RESULTS

Morphology

The colony and micro-morphologies of the novel *Aspergillus* strains 417 and 418 were identical. They most closely resembled those of *A. cretensis*. The following similarities were observed on CYA: superficial texture, conidial production, the extent of sclerotia production, the presence of abundant exudates in conspicuous droplets that were clear to pale yellow, the shape of the heads, and the ellipsoidal shape of the conidia. The most noticeable morphological differences between these two novel *Aspergillus* isolates and *A. cretensis* on CYA were that, in the former, the colony size was slightly smaller and, on MEA, the intensity and shades of colour were different and exudates absent. Moreover, the two novel *Aspergillus* isolates also had shorter and generally yellow-coloured stipes, larger vesicles, metulae, phialides and conidia, as well as smaller and regularly subglobose sclerotia.

The micro- and macromorphological differences between the two novel *Aspergillus* strains and *A. cretensis*, as well as other related species of the genus *Aspergillus* belonging to section *Circumdati*, pointed to the existence of a novel species, *Aspergillus affinis* sp. nov., described here (Fig. 1).

OTA production

When studied under experimental conditions, strains 417 and 418 gave a positive result in the OTA assay. When grown in CY broth, the novel strains showed the highest growth rate, largest increase in the concentration level of the synthesized mycotoxin and the greatest increase in the OTA production rate from the third to the fifth day of incubation (Fig. 2). A chromatogram of the extract from the culture filtrate (Fig. 2) yielded a peak corresponding to OTA in the extract from the 8-day-old CY broth culture.

Molecular and phylogenetic analysis

For *A. affinis* sp. nov. (see Table S1), the ~1000 nt obtained from the ID region were aligned with similar sequences extracted from GenBank. *A. affinis* sp. nov. strains 417 and 418 appeared invariant in the ID region sequences examined. The ITS-5.8S-ITS2 sequences (ca. 374 nt) of *A. affinis* sp. nov. examined were similar to those of the *A. cretensis* strains NRRL 35672^T and NRRL 35673 (Table S1), exhibiting a single indel in the ITS2 region. A high degree of similarity (99.3 %) emerged when *A. affinis* sp. nov. was compared with *A. westerdijkiae* (Table S1). A BLAST analysis of the 615-nucleotide region of the 28S rDNA gene of *A.*

affinis sp. nov. was conducted to compare the sequence with those available for *A. cretensis* strains. The comparison showed that *A. affinis* sp. nov. showed 100 % similarity to strain NRRL 35672^T (Table S1) and displayed a single transition (A→G) when compared with strain NRRL 35673. A high degree of similarity (ca. 99.5 %) also emerged when *A. affinis* sp. nov. was compared with *A. ochraceus*, *A. ostianus*, *A. melleus*, '*A. petrakii*' and *A. westerdijkiae* (Table S1). The NJ tree of the ID sequences (data not shown) demonstrated the presence of the main clades with a generally low level of bootstrap support (up to 86 %) and a general lack of phylogenetic resolution at the species level (e.g. *A. elegans* compared with *A. steynii*; *A. neobridgeri* compared with *A. roseoglobulosus*). The clade formed by *A. affinis* sp. nov. and *A. cretensis* (bootstrap value of 94 %) was found to be closely related to *A. westerdijkiae* (bootstrap value of 65 %).

Strains 417 and 418 of *A. affinis* sp. nov. showed identical sequences for a ~570-bp section of the *benA* gene, which contained three introns bounded by canonical GT-AG splice sites. Strains 417 and 418 and *A. cretensis* strains NRRL 35672^T and NRRL 35673 showed the same intron size, though a single deletion was observed in the former. Between *A. affinis* sp. nov. and *A. cretensis* the *benA* sequence divergence was ~3 % and this was mainly ascribable to the introns (around 12–13 changes). The exon sequences only displayed four different nucleotides that occurred as silent changes. When compared with other *Aspergillus* species of section *Circumdati*, a group of nine species, consisting of *A. affinis* sp. nov., *A. cretensis*, *A. melleus*, *A. muricatus*, *A. ochraceus*, *A. ostianus*, '*A. petrakii*', *A. westerdijkiae* and *Neopetromyces muricatus*, revealed some amino acid substitutions located in the region preceding the β -strand B2 (namely threonine→asparagine, threonine→serine and serine→alanine). Only the first two substitutions emerged when the group of nine species was compared with *A. auricomus* and *A. insulicola* (Table S1). The phylogenetic analysis conducted using the NJ and MP methods revealed the same tree topologies. A NJ cladogram of the partial β -tubulin gene is shown in Fig. 3, where most of the species of the genus *Aspergillus* examined resolved into well-supported clades. In the cluster formed by the previously mentioned nine species, *A. muricatus* and *N. muricatus* were the early-branching species (bootstrap value of 90 % in both NJ and MP trees) among the other species (Fig. 3). *A. affinis* sp. nov. was most closely related to *A. cretensis*, but was clearly distinct from the latter species (bootstrap value of 100 % in both NJ and MP trees; see Fig. 3). The Bayesian analysis of the *benA* gene sequences (data not shown) generally confirmed the results yielded by the NJ phylogenetic analysis (Fig. 3), including the observation that *A. affinis* sp. nov. and *A. cretensis* are two distinct evolutionary entities (1.00 posterior probability) and that *A. muricatus* and *N. muricatus* were identified as their basal lineages (1.00 posterior probability).

In addition, the *cmd* gene sequences (a total length of ca. 690 nt) obtained for *A. affinis* sp. nov. (Table S1) included

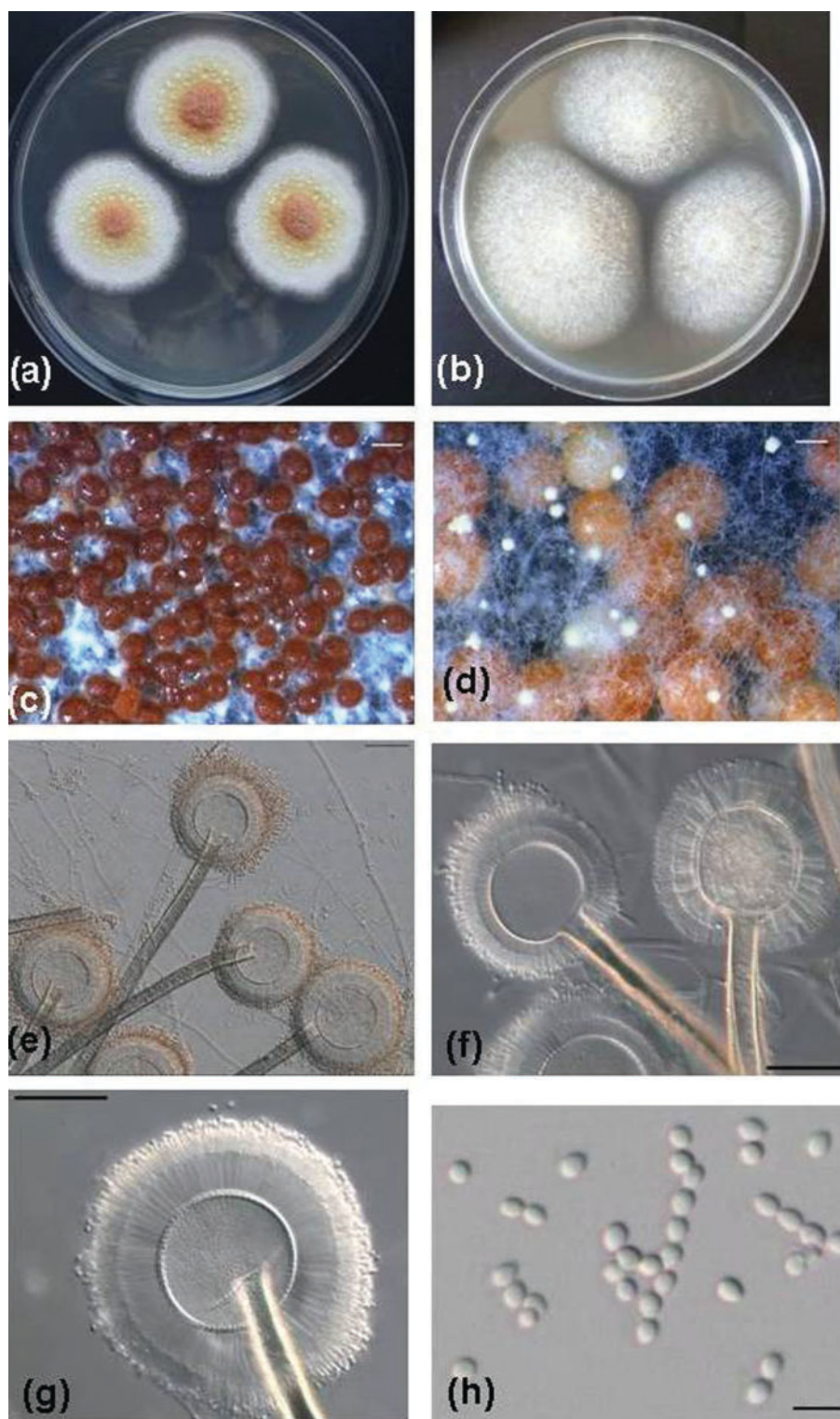


Figure 1. *A. affinis* sp. nov. ATCC MYA-4773^T (=CBS 129190=417). (a–b) Colonies grown on CYA (left) and MEA (right) at 25 °C, after 7 days; (c–d) sclerotia on CYA (left) and MEA (right) at 25 °C, after 15 days; (e–g) conidiophores; (h) conidia. (e–h), Nomarski interference contrast light microscopy. Bars, c, d, 500 µm; e, f, g, 50 µm; h, 6.25 µm.

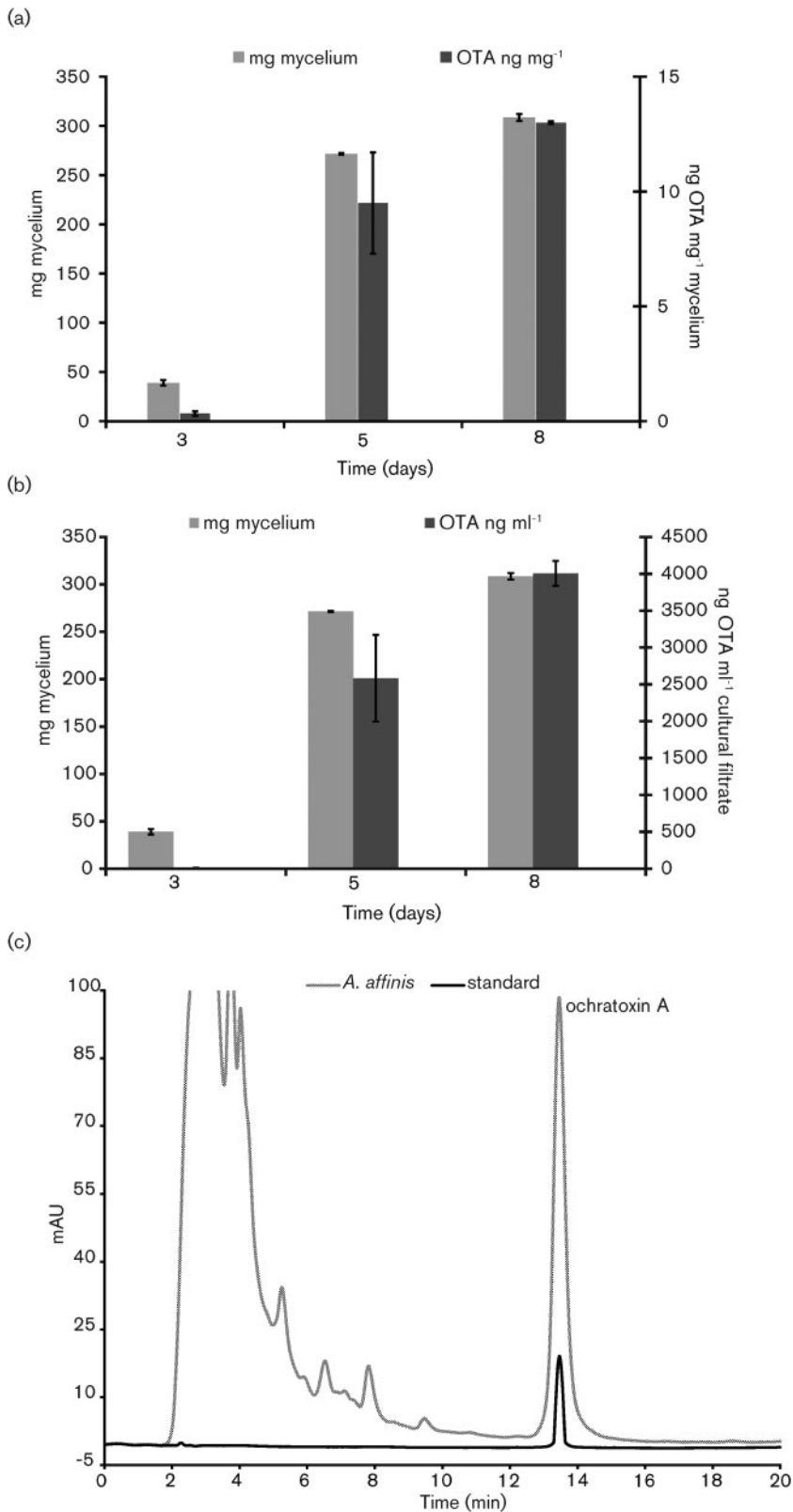


Fig. 2. *A. affinis* sp. nov. (a) Mycelial growth (expressed in mg) and OTA production (ng mg⁻¹ mycelium); (b) mycelial growth (expressed in mg) and OTA production (ng ml⁻¹ culture filtrate). The fungus was incubated for 8 days at 25 °C in CYA medium. Each result is the mean ± SD of three determinations; (c) HPLC chromatogram of the extract from the culture filtrate (see text) grown for 8 days at 25 °C in CYA medium (dotted lines) and of the OTA standard (continuous line).

four exons and three introns (bounded by the consensus sequences GT and AG, respectively). Identical *cmd* gene sequences emerged from the two isolates of *A. affinis* sp.

nov. A BLAST analysis for the *cmd* gene sequences of *A. affinis* sp. nov. displayed ca. 98 % sequence similarity with the sequence from the *A. cretensis* strains (Table S1). In the

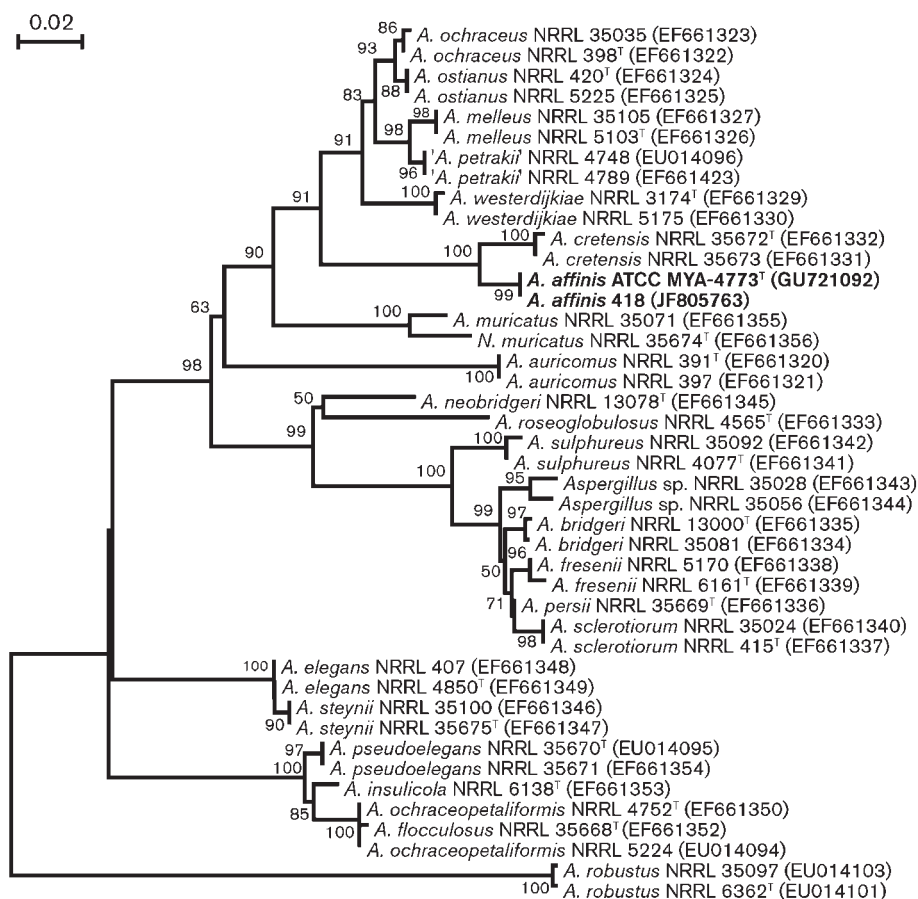


Fig. 3. Neighbour-joining tree of *A. affinis* sp. nov. strains ATCC MYA-4773^T (=CBS 129190=417) and 418, other examined *Aspergillus* taxa and the teleomorphic *N. muricatus* of section *Circumdati* based on the *benA* gene sequence data (GenBank accession nos in parentheses; sequences obtained in the present study are in bold type). The numbers near the branches are the bootstrap values (1000 pseudoreplicates); values <50% are not shown. Bar, 0.02 substitutions per nucleotide. Note: according to Peterson (2008), '*A. petrakii*' (strains NRRL 4748 and NRRL 4789) is a species name without standing in nomenclature under the rules of the ICBN, and strains NRRL 35028 and NRRL 35056 form an undescribed species of the genus *Aspergillus*.

cmd region examined, *A. affinis* sp. nov. displayed unique *cmd* gene sequences that distinguished it from *A. cretensis*. In particular, *A. affinis* sp. nov. and *A. cretensis* (Table S1) had different nucleotides, mainly in the intron regions (12 changes), whose length was the same. The exon sequences were nearly identical, revealing two different nucleotides that occurred as synonymous changes. None of the lineages belonging to section *Circumdati* exhibited any amino acid substitutions in the *cmd* region investigated in this study. The topology of the NJ tree obtained from the *cmd* gene sequences (Fig. S1) also suggested that *A. affinis* sp. nov. and *A. cretensis* were two clearly separate species (100% bootstrap support; Fig. S1). *A. muricatus* and *N. muricatus* emerged as basal taxa (bootstrap value 91%) to the cluster formed by *A. affinis* sp. nov., *A. cretensis*, *A. melleus*, *A. ochraceus*, *A. ostianus*, '*A. petrakii*' and *A.*

westerdijkiae. The Bayesian inference on the *cmd* gene sequences (data not shown) complemented the information yielded by the NJ phylogenetic analyses (Fig. S1) and confirmed the genetic separation between *A. affinis* sp. nov. and *A. cretensis* (1.00 posterior probability).

The phylogenetic analyses obtained from the combined sequences of the ID region, *benA* and *cmd* genes resulted in similar topologies; the NJ tree is shown in Fig. S2. *A. affinis* sp. nov. and *A. cretensis* were two distinct evolutionary entities (bootstrap value 100%; 1.00 posterior probability). Once again, *A. muricatus* and *N. muricatus* emerged as sisters (bootstrap value 99%, Fig. S2; 1.00 posterior probability) to the cluster formed by *A. affinis* sp. nov. and *A. cretensis*, as well as to a group including *A. melleus*, *A. ochraceus*, *A. ostianus*, '*A. petrakii*' and *A. westerdijkiae*.

DISCUSSION

In recent years, a growing interest in the estimation of freshwater microbial species diversity has promoted a more thorough investigation of aquatic microfungi. Detailed information, particularly at the species level, provides a basis for a better understanding of the ecological roles played in riparian plant litter degradation (Gulis *et al.*, 2008; Schneider *et al.*, 2010). Moreover, the effects of limited or missing data resulting from an insufficient taxonomic characterization of cryptic species may affect investigations aimed at estimating rates of species diversification. In this regard, the analysis of gene sequences has proved to be an important means of understanding evolutionary relationships at different taxonomic levels for genera such as *Aspergillus* and *Penicillium* and even of revealing recent evolutionary speciation among morphologically similar species (e.g. Klich *et al.*, 2003; Samson & Varga, 2009; Davolos *et al.*, 2012). On the other hand, species diversity may be overestimated owing to morphological variants of the same taxon if species identification is based solely on morphology.

Although the morphological features of *A. affinis* sp. nov. appear to be very similar to those of *A. cretensis*, a detailed analysis of these two species shows that they are morphologically distinguishable. Indeed, a macromorphological investigation revealed significant differences between *A. affinis* sp. nov. (Fig. 1) and *A. cretensis* in the growth and colour of the respective colonies. Moreover, other differences that emerge from a micromorphological analysis of *A. affinis* sp. nov. (including the length of vesicles, metulae, phialides and conidia, and size and shape of sclerotia) point to a clear distinction between this species and *A. cretensis*.

The HPLC data show that *A. affinis* sp. nov. is able to produce OTA, as previously found for *A. cretensis* and other species within the section *Circumdati* (Frisvad *et al.*, 2004). However, the level of OTA biosynthesis demonstrated by *A. affinis* sp. nov. (Fig. 2) appears to be considerably higher than that of other species (*A. elegans*, *A. ochraceus* and *A. westerdijkiae*) of the section *Circumdati* whose ability to produce OTA has been determined quantitatively (Gil-Serna *et al.*, 2011). Besides the section *Circumdati*, several species of other sections of the subgenus *Circumdati* produce this secondary metabolite, including some species assigned to the sections *Flavi* (Frisvad & Samson, 2000) and *Nigri* (Perrone *et al.*, 2008). The taxonomic use of some chemical characteristics, including OTA, has been proposed (Frisvad *et al.*, 2007). Although this study was not designed specifically to investigate OTA expression within an evolutionary framework of *Aspergillus* lineages, the production/lack of OTA (chemotype data from Frisvad *et al.*, 2004) within the section *Circumdati* examined here was not fully in agreement with the evolutionary relationships inferred for the species belonging to this section (Fig. 3; Figs S1–S2).

As regards the DNA sequence data obtained, the results from this study showed well-supported clades representing the main lineages within the section *Circumdati*, thereby

corroborating the findings from a previous molecular phylogenetic study (Peterson, 2008). In the same paper, several species, including *A. cretensis*, were found to be isogenic or to display a low degree of genetic variation at the loci examined (see also Fig. 3; Figs S1–S2). In our study, *A. affinis* sp. nov. isolates (Table S1) were invariant at the genes examined (Fig. 3; Figs S1–S2), probably owing to the fact that both strains were collected from the same river. As regards the ID region of *A. affinis* sp. nov., the sequences obtained in this study are similar to those of *A. cretensis* (Table S1). Thus, these two species cannot easily be discriminated on the basis of the ID data alone. It is known that, within the section *Circumdati*, allied species may share similar or identical sequences in this rRNA region (e.g. *A. elegans* and *A. steynii*, *A. neobridgeri* and *A. roseoglobulosus*). The analysis based on the ID sequences reveals few phylogenetically informative sites owing to the low variability of this rRNA region at that evolutionary scale (see Peterson, 2008). The situation is similar in other sections of the genus *Aspergillus*, e.g. sections *Clavati* (Varga *et al.*, 2007b) and *Nigri* (Perrone *et al.*, 2008), in which the ID region cannot reliably be used to distinguish the majority of the species assigned to a section.

A. affinis sp. nov. is, however, clearly distinct from *A. cretensis* in both the analysed β -tubulin and calmodulin gene regions (Fig. 3; Fig. S1), as the statistical significance unequivocally indicates. This confirms that such protein coding genes provide a considerable amount of phylogenetic information that may help to discriminate closely-related species of the genus *Aspergillus* (e.g. Varga *et al.*, 2007a). Indeed, the phylogenetic position of *A. affinis* sp. nov. is distinct within a strongly supported clade based on the *benA* gene sequences of nine species including *A. cretensis*, *A. melleus*, *A. muricatus*, *A. ochraceus*, *A. ostianus*, '*A. petrakii*', *A. westerdijkiae* and *N. muricatus* (Fig. 3). The results from this study are in agreement with those from other recent studies in which the *benA* gene sequences were found to be more variable than those of the ID region, often allowing even closely related *Aspergillus* lineages to be discriminated (e.g. Varga *et al.*, 2007b). It is also worth bearing in mind that the β -tubulin gene divergence value found between *A. affinis* sp. nov. and *A. cretensis* (ca. 3%) is slightly higher than those obtained from similar sequences between other phylogenetically closely related *Aspergillus* species of the section *Circumdati* (ca. 0.6–2.6%). Intriguingly, the amino acid substitutions found in the region preceding the β -strand B2 (see Nogales *et al.*, 1998) for a clade of nine species consisting of *A. affinis*, *A. cretensis*, *A. ochraceus*, *A. ostianus*, *A. melleus*, *A. muricatus*, '*A. petrakii*', *A. westerdijkiae* and *N. muricatus* may be considered apomorphic molecular traits. This points to a molecular divergence between this group and other *Aspergillus* species of the section *Circumdati* (Fig. 3; Figs S1–S2).

In addition, in the *cmd* region examined in this study *A. affinis* sp. nov. and *A. cretensis* emerge as two distinct species, as the statistical analyses clearly indicate (Fig. S1); the *cmd* gene sequence similarity is around 98%, a value

that falls within the range of genetic variation between other evolutionarily closely related *Aspergillus* species of the section *Circumdati* obtained from similar sequences (96.5–100%). The *cmd* gene proved to be appropriate for the phylogenetic analysis performed in this study, as previously reported for other sections (Houbraken *et al.*, 2007; Perrone *et al.*, 2007, 2008; Peterson, 2008; Pildain *et al.*, 2008). When the *benA* and *cmd* markers were compared, these two genes were found to yield similar phylogenetic topologies (Fig. 3; Fig. S1).

Lastly, *A. affinis* sp. nov. is supported as an independent evolutionary lineage by very high statistical values in the phylogenetic trees even when the ID, *benA* and *cmd* gene sequences are pooled (Supplementary Fig. S2). Indeed, the combined data provide results that are comparable to those provided by the separate *benA* and *cmd* loci analyses, though with even greater statistical significance at the phylogenetic tree nodes, and with generally higher statistical values when a Bayesian computation is adopted. Moreover, the phylogenetic position of *A. affinis* sp. nov. was concordant in all the trees (Fig. 3; Figs S1–S2), which is in keeping with the phylogenetic species recognition concept (see Taylor *et al.*, 2000).

To sum up, the approach adopted in this study provides strong evidence suggesting that the evolution of *A. affinis* sp. nov. diverges from that of other species of the genus *Aspergillus*, insofar as the former displays peculiar morphological and ecological features as well as unique DNA sequences at multiple loci. The morphological data and the phylogenetic results indicate that *A. affinis* sp. nov. and *A. cretensis* are two closely related, though distinct, species (Fig. 3; Figs S1–S2). Thus, the morphological similarities shared by these two *Aspergillus* species reflect their close phylogenetic relationships. Indeed, the low genetic divergence values that emerged from this study suggest that these two species probably have a shared recent ancestry. Analyses of the coding regions of the β -tubulin and calmodulin genes (similar exon sequences), in particular, point to a recent speciation event within this monophyletic group (Fig. 3; Figs S1–S2). Accurate geographical sampling is warranted to determine whether these two species are restricted to certain regions in the Mediterranean Basin. Nonetheless, the ecological data that are currently available provide an informative perspective regarding the speciation of *A. affinis* sp. nov.

TAXONOMY

Latin diagnosis of *Aspergillus affinis* Davolos, Persiani, Pietrangeli et Maggi sp. nov.

Coloniae in agar CYA crescentes, post 7 dies 25 °C 25–30 mm diam, copiosis sclerotiis compositae, primo albis usque leviter gilvis, deinde leviter fuscantibus usque rubro-fuscis post 14–18 dies (M. 9D6–8); *mycelium album; conidiogenesis moderata, leviter gilva usque flavens* (M.

3A5–6); *Exudatum album usque pallens* (M. 3A4). *Facies reversa gilva* (M. 4A3) *usque leviter lutea* (M. 5A4); *solubile pigmentum absens. Coloniae in agar MEA crescentes, post 7 dies 25 °C 35–40 mm diam, mycelium album, conidiogenesis moderata, gilva usque pallens* (M. 2A3–4); *sclerotia primo alba deinde leviter lutea usque fusco-lutea* (M. 6A4–5); *exudatum absens; facies reversa alba usque pallens* (M. 3A2–3). *Coloniae in agar G25N crescentes, post 7 dies 25 °C 18–20 mm diam; mycelium album, floccosum; facies reversa leviter gilva; solubile pigmentum absens; conidiogenesis copiosa, sclerotia absunt. Incrementum nullum post 7 dies ad 5 °C et 37 °C in CYA. Capitula conidica radiantia; stipites plerumque longi 3500 μ m et lati 12.5–21.5 μ m, rugosi, flaventes; vesiculae globosae (57–79 μ m), sterigmata duoseriata, primaria 9–18 $\times$ 4.5–5.4 μ m; secundaria plerumque 10.8–12.6 $\times$ 2.1–3.2 μ m; conidia enodia vel leviter rugosa plerumque 3.9–4.5 $\times$ 2.7–3.6 μ m; sclerotia copiosa, globosa vel subglobosa, 500–800 $\times$ 300–600 μ m. Status teleomorphosis ignotus.*

Typus ATCC MYA-4773^T (=CBS 129190=417). MycoBank MB517245.

Description of *A. affinis* Davolos, Persiani, Pietrangeli and Maggi sp. nov. (Fig. 1)

Etymology: from the Latin word *affinis* which means ‘similar, related’ and reflects the close morphological similarity of the novel species to *A. cretensis*.

Colony diameter after 7 days on CYA at 25 °C is 25–30 mm, dominated by abundant, white to light yellow sclerotia, becoming light brown to reddish brown after 14–18 days (colour reference code M. 9D6–8). Mycelium white; very sparse conidial production on CYA is light yellow to yellow (M. 3A5–6). Exudate white to pastel yellow (M. 3A4). Reverse pale yellow (M. 4A3) to light orange (M. 5A4); no soluble pigments present. Colony diameter after 7 days on MEA at 25 °C is 35–40 mm, floccose; mycelium white; sparse conidial production, pale yellow to pastel yellow (M. 2A3–4); sclerotia formed, white to light orange (M. 6A4–5) up to brownish orange (M. 6C6–8); exudate absent; reverse yellowish white to pastel yellow (3A2–3). Colonies after 7 days on G25N at 25 °C 18–20 mm diameter, mycelium white, floccose; reverse pale yellow; no soluble pigment present; good conidial production; sclerotia have not been observed. No growth on CYA at 5 °C and 37 °C. Conidial heads radiate; stipes long, up to 3500 μ m long, 12.5–21.5 μ m wide, walls rough, yellow pigmented; vesicles large, globose (57–79 μ m), biserial; metulae covering the entire vesicle, measuring 9–18 $\times$ 4.5–5.4 μ m; phialides 10.8–12.6 $\times$ 2.1–3.2 μ m; conidia predominantly ellipsoidal, smooth to finely roughened, 3.9–4.5 $\times$ 2.7–3.6 μ m; sclerotia abundant, globose to subglobose, 500–800 $\times$ 300–600 μ m. Not known to produce a teleomorphic state.

This species is morphologically related to *A. cretensis* but differs from the latter in colony growth and colour, in the

size of the vesicles, metulae, phialides and conidia, which are larger, and in the sclerotia, which are smaller and regularly subglobose.

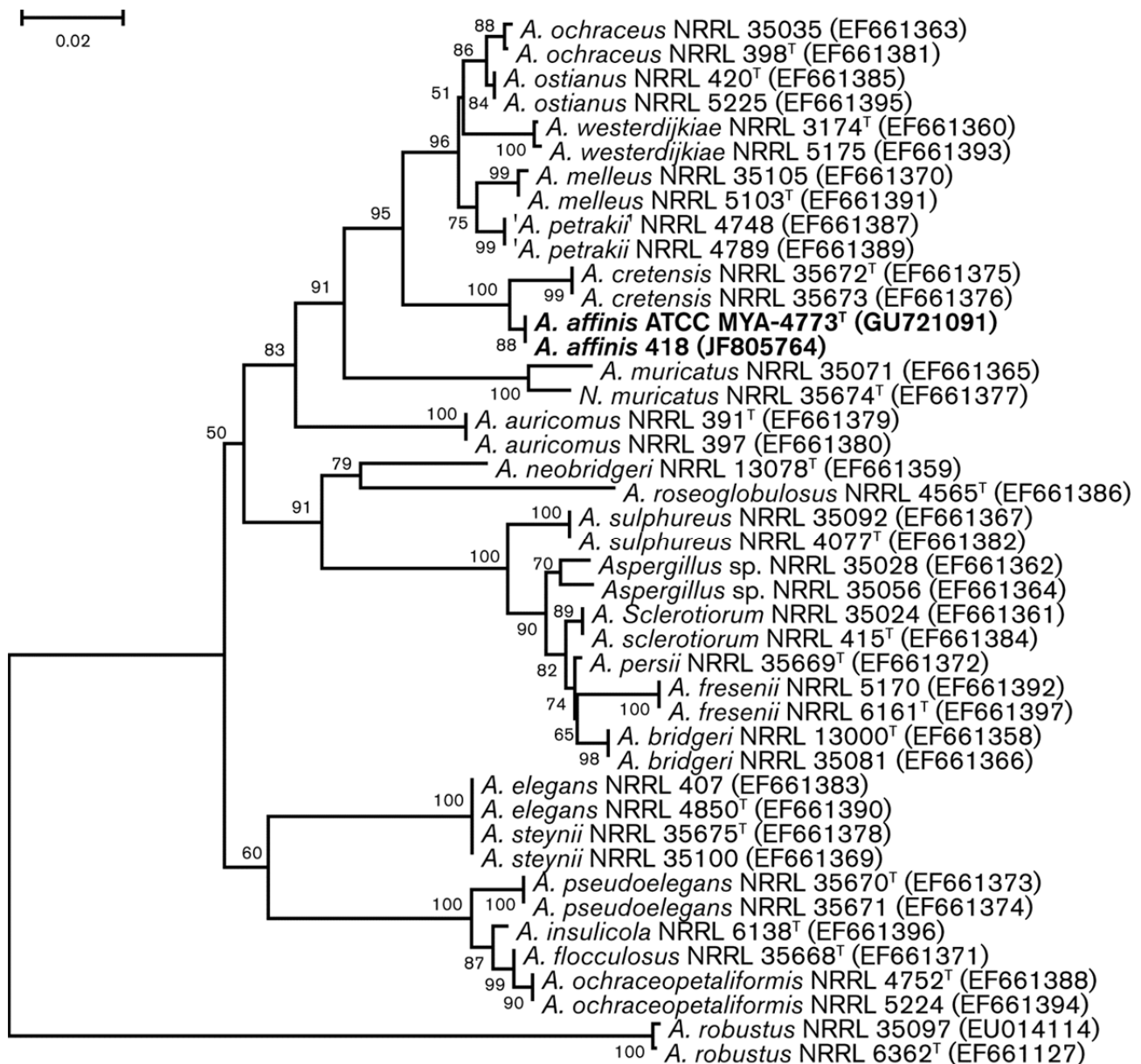
The type strain is ATCC MYA-4773^T (=CBS 129190 =417; deposited in the Department of Environmental Biology, Sapienza University of Rome, Rome, Italy). Strain 418, an additional isolate, was deposited in the Department of Environmental Biology, Sapienza University of Rome, Rome, Italy. MycoBank accession no. MB517245.

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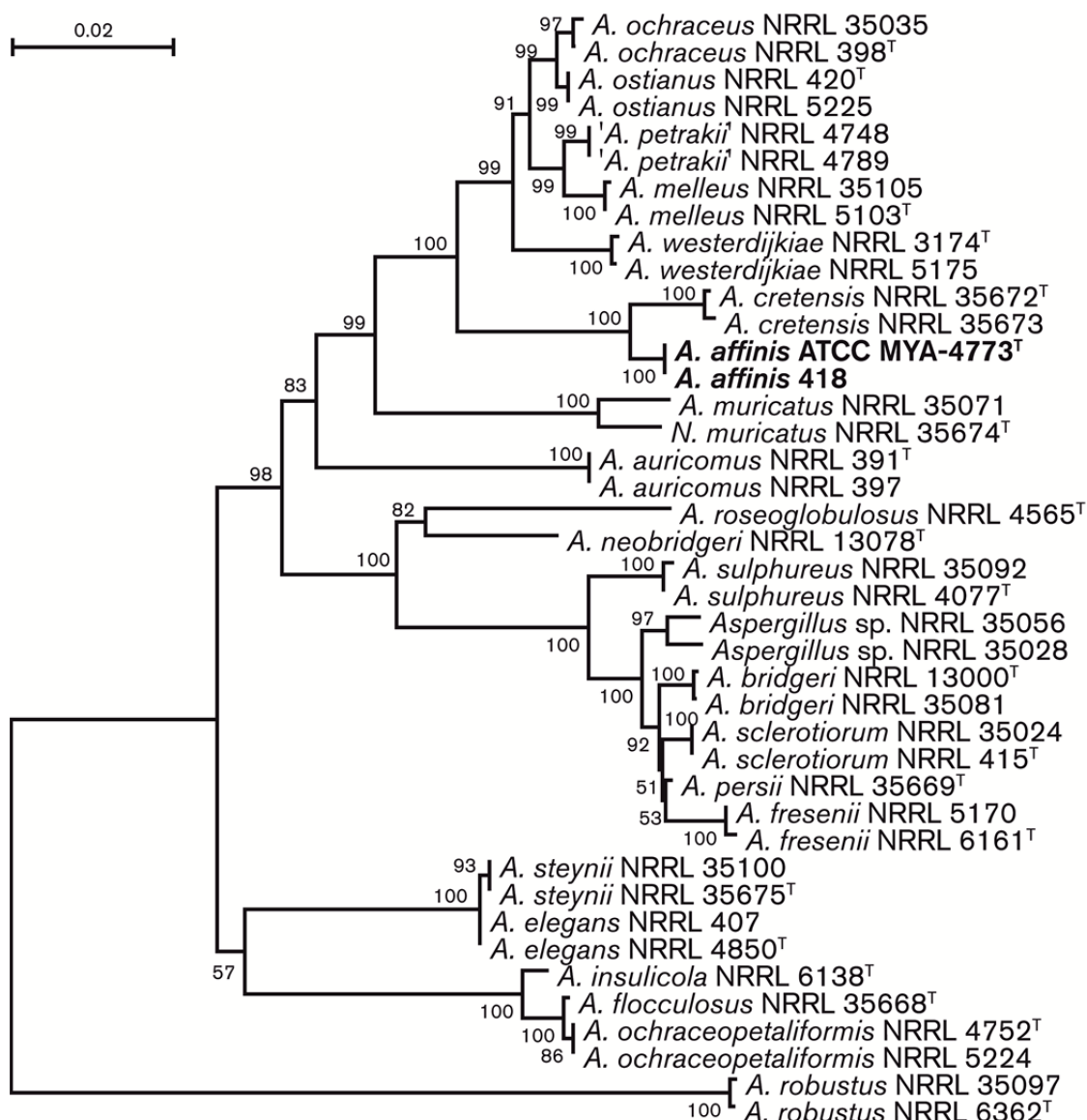
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Supplementary Figure S1. Neighbour-joining tree of *A. affinis* sp. nov. strains ATCC MYA-4773^T (=CBS 129190^T=417^T) and 418, other examined *Aspergillus* taxa of section *Circumdati* and *Neopetromyces muricatus* based on the *cmd* gene sequence data (GenBank accession nos are shown in parentheses; the sequences obtained in the present study are in bold type). The numbers near the branches are the bootstrap values (1000 pseudoreplicates). Bar, 0.02 substitutions per nucleotide. Note: according to Peterson (2008), '*A. petrakii*' (strains NRRL 4748 and NRRL 4789) is a name without standing in nomenclature under the rules of the ICBN, and the strains NRRL 35028 and NRRL 35056 form an undescribed species of the genus *Aspergillus*.



Supplementary Figure S2. Neighbour-joining tree of *A. affinis* sp. nov. and other lineages of section *Circumdati* based on the pooled ID region, *benA* and *cmd* gene sequence data (see text; the DNA sequences obtained in the present study are highlighted in bold; for the GenBank accession nos see Supplementary Table S1). *A. pseudoelegans* strains NRRL 35670^T and NRRL 35671 were not included in this phylogenetic analysis because, at the time of writing, only DNA sequences from the 28S rDNA gene of the rRNA genes were available from GenBank. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The numbers near the branches are the bootstrap values (1000 pseudoreplicates). Note: according to Peterson (2008), '*A. petrakii*' (strains NRRL 4748 and NRRL 4789) is a name without standing in nomenclature under the rules of the ICBN, and the strains NRRL 35028 and NRRL 35056 form an undescribed species of the genus *Aspergillus*.

Supplementary Table S1. List of *A. affinis* and other examined lineages of section *Circumdati* included in the molecular phylogenetic analysis of the present study, GenBank accession numbers and length in base pair (bp) of the ID region (unless otherwise stated), of the *benA* and of the *cmd* sequences (see text)

Lineage	ID	bp	<i>benA</i>	bp	<i>cmd</i>	bp
<i>Aspergillus affinis</i> ATCC MYA-4773 ^T =CBS 129190 ^T =417 ^T	GU721090	988	GU721092	568	GU721091	689
<i>Aspergillus affinis</i> 418	JF805762	973	JF805763	567	JF805764	688
<i>Aspergillus auricomus</i> NRRL 391 ^T	EF661411	1143	EF661320	538	EF661379	780
<i>Aspergillus auricomus</i> NRRL 397	EF661412	1143	EF661321	538	EF661380	780
<i>Aspergillus bridgeri</i> NRRL 13000 ^T	EF661404	1158	EF661335	524	EF661358	777
<i>Aspergillus bridgeri</i> NRRL 35081	EF661398	1157	EF661334	524	EF661366	777
<i>Aspergillus cretensis</i> NRRL 35672 ^T	EF661417	1150	EF661332*	542	EF661375	786
<i>Aspergillus cretensis</i> NRRL 35673	EF661418	1150	EF661331	542	EF661376	786
<i>Aspergillus elegans</i> NRRL 4850 ^T	EF661414	1146	EF661349	540	EF661390	774
<i>Aspergillus elegans</i> NRRL 407	EF661413	1146	EF661348	540	EF661383	774
<i>Aspergillus flocculosus</i> NRRL 35668 ^T †	EF661432	1151	EF661352	543	EF661371	771
<i>Aspergillus fresenii</i> NRRL 6161 ^T	EF661403	1156	EF661339	525	EF661397	778
<i>Aspergillus fresenii</i> NRRL 5170	EF661402	1156	EF661338	525	EF661392	778
<i>Aspergillus insulicola</i> NRRL 6138 ^T	EF661430	1161	EF661353	544	EF661396	771
<i>Aspergillus melleus</i> NRRL 5103 ^T	EF661425	1130	EF661326	546	EF661391	782
<i>Aspergillus melleus</i> NRRL 35105	EF661426	1133	EF661327	546	EF661370	782
<i>Aspergillus muricatus</i> NRRL 35071	EF661433	1159	EF661355	536	EF661365	786
<i>Aspergillus neobridgeri</i> NRRL 13078 ^T	EF661410	1156	EF661345	527	EF661359	776
<i>Aspergillus ochraceopetaliformis</i> NRRL 4752 ^T †	EF661429	1151	EF661350	543	EF661388	771
<i>Aspergillus ochraceopetaliformis</i> NRRL 5224†	EU021616	1163	EU014094	543	EF661394	771
<i>Aspergillus ochraceus</i> NRRL 398 ^T	EF661419	1134	EF661322	542	EF661381	783
<i>Aspergillus ochraceus</i> NRRL 35035	EF661420	1134	EF661323	542	EF661363	783
<i>Aspergillus ostianus</i> NRRL 420 ^T	EF661421	1133	EF661324	541	EF661385	787
<i>Aspergillus ostianus</i> NRRL 5225	EF661422	1133	EF661325	541	EF661395	760
<i>Aspergillus persii</i> NRRL 35669 ^T	EF661399	1157	EF661336	524	EF661372	778
‘ <i>Aspergillus petrakii</i> ’ NRRL 4748†	EU021613	1144	EU014096	546	EF661387	781
‘ <i>Aspergillus petrakii</i> ’ NRRL 4789†	EF661423	1132	EF661423	546	EF661389	781
<i>Aspergillus pseudoelegans</i> NRRL 35670 ^T	EU021606	771‡	EU014095	543	EF661373	769
<i>Aspergillus pseudoelegans</i> NRRL 35671	EU021607	530‡	EF661354	543	EF661374	769
<i>Aspergillus robustus</i> NRRL 6362 ^T	EF661176	1129	EU014101	506	EF661127	763
<i>Aspergillus robustus</i> NRRL 35097	EF661435	1129	EU014103	505	EU014114	768
<i>Aspergillus roseoglobulosus</i> NRRL 4565 ^T	EF661406	1163	EF661333	525	EF661386	753
<i>Aspergillus sclerotiorum</i> NRRL 415 ^T	EF661400	1156	EF661337	524	EF661384	777
<i>Aspergillus sclerotiorum</i> NRRL 35024	EF661401	1156	EF661340	524	EF661361	777
<i>Aspergillus steynii</i> NRRL 35675 ^T	EF661416	1149	EF661347	539	EF661378	774
<i>Aspergillus steynii</i> NRRL 35100	EF661415	1149	EF661346	539	EF661369	774

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Supplementary Table S1 ctd.

Lineage	ID	bp	<i>benA</i>	bp	<i>cmd</i>	bp
<i>Aspergillus sulphureus</i> NRRL 4077 ^T	EF661409	1156	EF661341	524	EF661382	772
<i>Aspergillus sulphureus</i> NRRL 35092	EF661408	1160	EF661342	524	EF661367	775
<i>Aspergillus westerdijkiae</i> NRRL 3174 ^T	EF661427	1132	EF661329	536	EF661360	774
<i>Aspergillus westerdijkiae</i> NRRL 5175	EF661428	1132	EF661330	537	EF661393	774
<i>Aspergillus</i> sp. NRRL 35028	EF661407	1163	EF661343	523	EF661362	776
<i>Aspergillus</i> sp. NRRL 35056	EF661405	1163	EF661344	523	EF661364	776
<i>Neopetromyces muricatus</i> NRRL 35674 ^T	EF661434	1159	EF661356	540	EF661377	784

*The *benA* gene sequences obtained from the type strain of *A. cretensis* by Frisvad *et al.* (2004) and by Peterson (2008), respectively AY819977 and EF661332, display two base pair differences. We used the latter data alone because all the sequences extracted from GenBank and examined in our study were from Peterson (2008).

†According to Peterson (2008), *A. flocculosus* is conspecific with *A. ochraceopetaliformis*, and '*A. petrakii*' is a name without standing in nomenclature under the rules of the ICBN.

‡It corresponds solely to one region of the 28S rDNA gene.

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