RESEARCH ARTICLE

β-tubulin paralogue *tubC* is frequently misidentified as the *benA* gene in *Aspergillus* section *Nigri* taxonomy: primer specificity testing and taxonomic consequences

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Abstract   β-tubulin (*benA*, *tub-2*) and calmodulin (*caM*) are crucial genes in the taxonomy of *Aspergillus* section *Nigri*. Widely used β-tubulin primers are not specific for the *benA* gene for some taxa and preferentially amplify the *tubC* paralogue. Sequences of the *tubC* paralogue are widely combined with *benA* sequences in recent taxonomical works as well as other works, resulting in incongruent trees. In this study we newly provide *benA* sequences for several ex-type strains, which were characterised using the *tubC* gene only. We designed a highly specific forward primer to *benA* designated *Ben2f* for use in *Aspergillus* section *Nigri*, and tested specificity of numerous primer combinations to β-tubulin paralogs. The primer pairs with the highest specificity to the *benA* gene and functional across species in section *Nigri* includes *Ben2f/Bt2b*, *Ben2f/T22* and *T10/T22*. We also provide tools based on codon usage bias analysis that reliably distinguish both paralogues. Exon/intron arrangement is the next distinctive characteristic, although this tool is not valid outside section *Nigri*. The species identity of taxa from the 'A. aculeatus clade' used in previous molecular studies was revised using combined molecular data (ITS, *benA*, *caM*). These data together with two different PCR-fingerprinting methods indicated that *A. japonicus* should be treated as a synonym of *A. violaceofuscus*. Similarly, *A. fijiensis* is reduced to synonymy with *A. brunneoviolaceus*.

INTRODUCTION

Aspergillus section *Nigri* includes biotechnologically, economically and medically important organisms. Some species produce important organic acids or enzymes. Others are known to cause food spoilage or severe human infections. There are 26 species distinguished based on the polyphasic approach (Varga et al. 2011). A number of schemes have been proposed for classification and identification of black aspergilli. Molecular approaches have revealed high diversity among species that are difficult to recognise based solely on their phenotypic characters (Samson et al. 2007, Varga et al. 2011). The ITS sequence offers only low discrimination level to major clades. The *benA* and *caM* genes are the most informative loci in classification of black aspergilli and are broadly preferred by taxonomists and in routine identification. Despite this, the *benA* or *caM* genes alone are not able to distinguish all species in section *Nigri* (Varga et al. 2011). Thus, the polyphasic approach recommended for description of new *Aspergillus* species (Samson & Varga 2009) is only employed in a limited fashion in section *Nigri*.

Paralogous genes represent well-known problems in taxonomy that uses molecular features as the indispensable tool for delineation of taxa. The origins of paralogous genes are most frequently attributed to gene duplication. Among members of the tubulin superfamily, only α-, β- and γ-tubulins have homologues in fungal genomes (Dutcher 2001). β-tubulin paralogues are represented in *Aspergillus* by two genes designated *benA* and *tubC*. Gene *benA* (*tub-2*) encodes polypeptides designated as β1- and β2-tubulin (diversifying due to different post-transcriptional modifications) and is the third most utilised gene in fungal multilocus phylogenies (Feau et al. 2011). Polypeptide β3-tubulin (Weatherbee & Morris 1984) is encoded by the *tubC* paralogue and was first described in *A. nidulans* (Weatherbee et al. 1985). Whereas *benA* is a housekeeping gene, *tubC* is not essential for growth and is expressed only under specific conditions (see Discussion). In this study, we show that β-tubulin paralogues are commonly mixed in taxonomical studies producing incongruent phylogenetic trees and bringing discrepancies into the taxonomy of black aspergilli.

MATERIAL AND METHODS

Molecular studies

DNA was extracted from 7 d old colonies using the Microbial DNA Isolation Kit (Mo-Bio Laboratories, Inc.). For phylogenetic analysis, the ITS region, partial *benA* gene and partial *caM* gene were chosen because they were used in recent taxonomical monographs (Samson et al. 2004, 2007, Varga et al. 2011). The Mastercycler Gradient (Eppendorf) was used to amplify the desired regions. The ITS region of the rDNA was amplified using primers *ITS1F* and *NL4* (O'Donnell 1992, Gardes & Bruns 1993). The partial *caM* gene sequences were amplified with primers *CF1M* or *CF1L* and *CF4* as described by Peterson (2008). PCR amplification was performed using the 'Type I' conditions (see below). The *benA* and *tubC* loci were amplified using primers listed below in section 'Primer specificity testing' and amplified using two different cycling conditions. The PCR product purification and sequencing was provided by Macrogen Europe, The Netherlands. DNA sequences obtained in this study were deposited in the EMBL database under the accession numbers listed in Table 1, while other sequences are deposited under numbers HE818079–HE818087.

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Table 1   List of isolates from section *Nigri* that were used for primer testing.

	species and isolate number	benA (Ben2f/Bt2b)	tubC (Bt2a/Bt2b)	caM	ITS
A	*A. violaceofuscus* CBS 114.51[1]	**HE577804**	**HE577812**	AJ964875	AJ279985
B	*A. violaceofuscus* CBS 123.27[NT]	**HE577805**	**HE577813**	FJ491698	FJ491678
C	*A. aculeatus* CBS 172.66[T]	**HE577806**	**HE577814**	AJ964877	AJ279988
D	*A. brunneoviolaceus* CCF 108	**FR775311**	**HE577818**	**HE608868**	**FR727129**
E	*A. aculeatinus* F-596	**HE577809**	no product	**HE578095**	**HE578070**
F	*A. aculeatus* F-719	**HE577810**	**HE577816**	**HE578093**	**HE578071**
G	*Aspergillus* sp. CCF 4046	**HE577817**	**HE577815**	**HE578097**	**HE578072**
H	*A. violaceofuscus* CCF 4079	**HE577811**	not sequenced[2]	**FR751423**	**FR733805**
I	*A. tubingensis* CCF 2818	**HE577808**	no product	**FR751416**	**FR727132**
J	*A. niger* CCF 3990	**FR775364**	no product	**FR751421**	**FR727126**
K	*A. carbonarius* CCF 3388	**HE577803**	no product	**HE649500**	**FR727127**
L	*A. piperis* CCF 661	**HE577807**	no product	**FR751415**	**FR733803**

[1]   ex-type of *A. japonicus*.
[2]   The product was identified as *tubC* based on the similar length of the product to the *tubC* from the ex-neotype culture of *A. violaceofuscus* (Fig. 3).
Sequences that originated from this study are in **bold** print.

Ben2f primer design

Primer *Ben2f* (*benA* specific, exon nr. 2, forward; 5´-TCCAGAC-TGGTCAGTGTGTAA) was designed based on the complete *benA* sequences of *A. niger*, *A. carbonarius* and *A. aculeatus* (The US Department of Energy Joint Genome Institute http://www.jgi.doe.gov). The primer was designed using Primer3Plus software (Untergasser et al. 2007). The obtained primers that matched with the *tubC* paralogue sequences of aspergilli with known genome sequences (Askenazi et al. 2003, Galagan et al. 2005, Payne et al. 2006, Wortman et al. 2006) were rejected.

Primer specificity testing

The *Bt2a* (5´-GGTAACCAAATCGGTGCTGCTTTC), *T10* (5´-ACGATAGGTTCACCTCCAGAC) and *Ben2f* (5´-TCCA-GACTGGTCAGTGTGTAA) were selected as forward primers; *Bt2b* (5´-ACCCTCAGTGTAGTGACCCTTGGC), *T224* (5´-GAGGGAACGACGGAGAAGGTG), *T222* (5´-GACCGGG-GAAACGGAGACAGG) and *T22* (5´-TCTGGATGTTGTTGG-GAATCC) were selected as reverse primers for testing. The position of all primers is indicated on Fig. 1. The specificity of all possible primer combinations was tested using two different PCR cycling conditions. The identity of amplification products was verified by sequencing or by length of fragments observed on the electrophoretograms. The mixture (25 µL) contained 50 ng of genomic DNA, 20 pmol of each primer, 0.2 mM of dNTPs, and 1 U of PerfectTaq DNA polymerase with the respective buffer. On an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg), two types PCR cycling condition were used. The ˙Type I˙ conditions comprised 32 cycles under the following temperature regime: 95 °C/3 min, 55 °C/30 s, and 72 °C/1 min (1×), 95 °C/30 s, 55 °C/30 s, and 72 °C/1 min (30×) and 95 °C/30 s, 55 °C/30 s, and 72 °C/10 min (1×). In case of primer combinations with T22 primer, the extension time 90 s was used. The ˙Type II˙ touchdown cycling conditions involved an initial 2 min denaturation step at 93 °C, followed by 5 cycles in which the DNA samples were denatured at 93 °C for 30 s and annealed for 30 s with a decrease in 1 °C in each successive cycle. The regime started with an annealing temperature 65 °C decreasing to 60 °C. The extension was proceeded at 72 °C for 1 min (in case of primer combinations with T22 primer, the extension time 90 s was used). Annealing at the 60 °C was then used for further 33 cycles with a final extension for 10 min.

Phylogenetic analysis

Sequences were inspected and assembled using the Bioedit sequence alignment editor v. 7.0.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Alignments of the regions were performed using the FFT-NSi strategy as implemented in MAFFT v. 6.861b (Katoh et al. 2005).

The phylogram is shown on Fig. 2 and includes all β-tubulin sequences of taxa belonging to the *A. aculeatus* clade and *benA* sequences of other species from section *Nigri* from GenBank. The introns were extracted from sequences using features for *benA* and *tubC* paralogues published by May et al. (1987) (reference annotated sequences: M17519 for *benA* and M17520 for *tubC*). The maximum likelihood (ML) method implemented in MEGA5 (Tamura et al. 2011) was used with settings similar to that for the phylograms in Fig. 3 (see below). In addition, the codon positions were indicated and all were included in the analysis. The tree with the highest log likelihood is shown (Fig. 2). There were a total of 207 positions (73 variables) in the final dataset.

The ITS region and partial *benA* and *caM* sequences were combined. There were a total of 1 346 positions in the final dataset (430 variables). The evolutionary history was inferred by using the ML. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The number of bootstrap replicates was set to 500. *Aspergillus robustus*
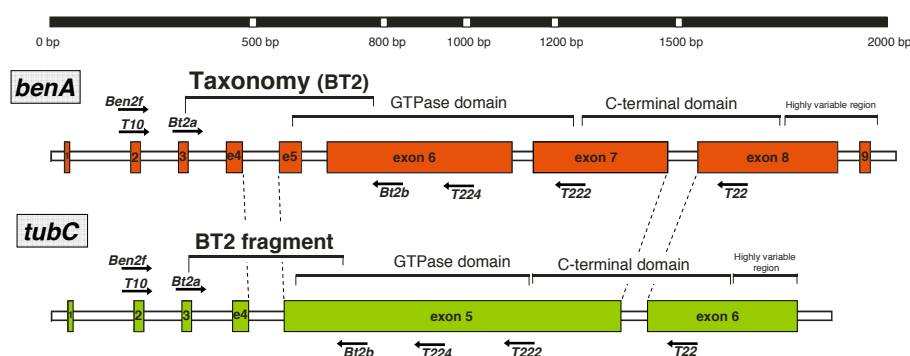

Fig. 1   The exon-intron arrangement of *benA* and *tubC* gene of *A. aculeatus*. The exons are in red (*benA*) and green (*tubC*) colour. The position of primers used for specificity testing (see Table 4) and their orientation is designated by arrows above and below exons.

NRRL 6362 was used as an outgroup. The tree with the highest log likelihood is shown (Fig. 3). The Bayesian tree inference analysis was used to calculate the posterior probabilities of branches (Huelsenbeck & Ronquist 2001). Separate partitions were created for exons and introns (*benA* and *caM*), ITS spacers and 5.8S rDNA. The exons were analysed allowing codon positions to be independent datasets. A general time reversible model was used with gamma-distributed rate variation across sites allowing six different types of substitutions. The MCMC analysis with 8×10^7 generations was run with two parallel chains incrementally heated by a temperature of 0.7, starting from a random tree. One tree was saved per 1 000 generations, and the run was ended when the likelihood scores of sampled trees approached similar values. A burn-in and convergence of the chains were determined with Tracer v1.4.1 (http://tree. bio.ed.ac.uk/software/tracer). Individual ML trees containing all sequences of *benA* (410 sites, 82 variables), *tubC* (298 sites, 83 variables) and *caM* (421 sites, 111 variables) were constructed from all sequences of taxa belonging to the *A. aculeatus* clade deposited at GenBank (until 11 May 2012) (Fig. 3). *Aspergillus*

saccharolyticus CBS 127449 was used as the outgroup for the *caM* and *benA* trees and *Emericella nidulans* (M17520) for the *tubC* tree.

PCR fingerprinting

The PCR fingerprinting with the phage M13-core sequence as an oligonucleotide primer (5´-GAGGGTGGCGGTTCT) was performed in 18.5 μL volumes, each contained 100 ng of DNA, 25 mM of MgCl$_2$ (Promega Corp.), 0.4 mM of dNTPs (Promega Corp.), 1 U of PerfectTaq DNA polymerase (5Prime) with the respective buffer and 20 pmol of M13-core primer. The reaction mixtures were subjected to 32 cycles under the following temperature regime: 94 °C/3 min, 52 °C/1 min, and 65 °C/3 min (1×); 45 °C/40 s, 52 °C/1 min, and 65 °C/3 min (35×) and 94 °C/40 s, 52 °C/1 min, and 65 °C/10 min (1×). The fingerprinting with primer 834t [(AG)$_8$CG] was performed in 18.5 μL volumes. The mixture contained 100 ng of DNA, 25 mM of MgCl$_2$, 0.3 mM of dNTPs, 10 mg of bovine serum albumin (MBI Fermentas), 1.5 M of betaine (Sigma), 1 U of PerfectTaq DNA polymerase with respective buffer and 10 pmol of 834t primer.
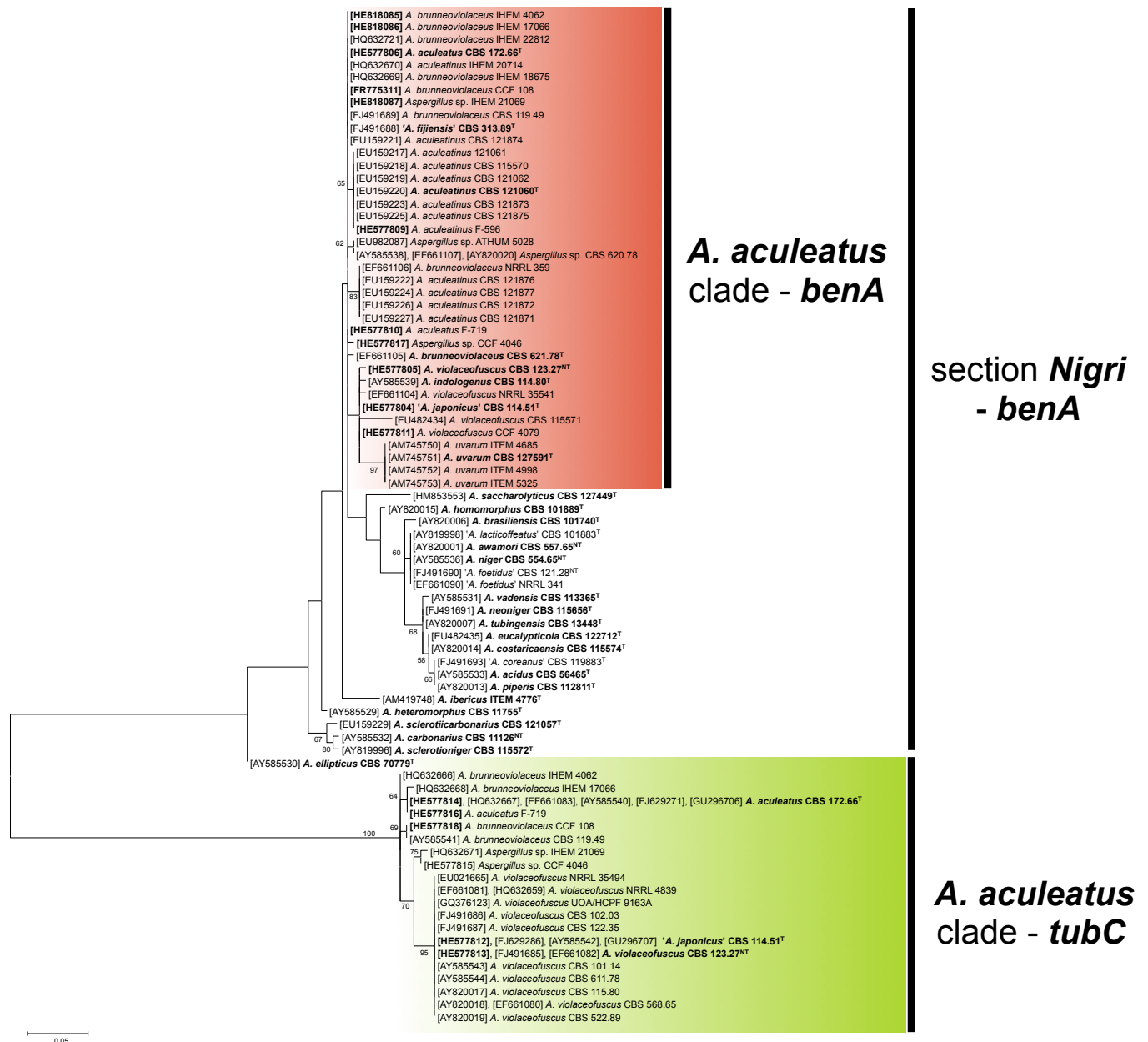

Fig. 2 The ML tree showing two distinct clusters among β-tubulin sequences of taxa belonging to *A. aculeatus* clade that were deposited in GenBank. The introns were removed from the alignment. Only bootstrap values > 60 % are shown. The accession numbers of sequences deposited in this study and the names of the type specimens are in **bold** print.
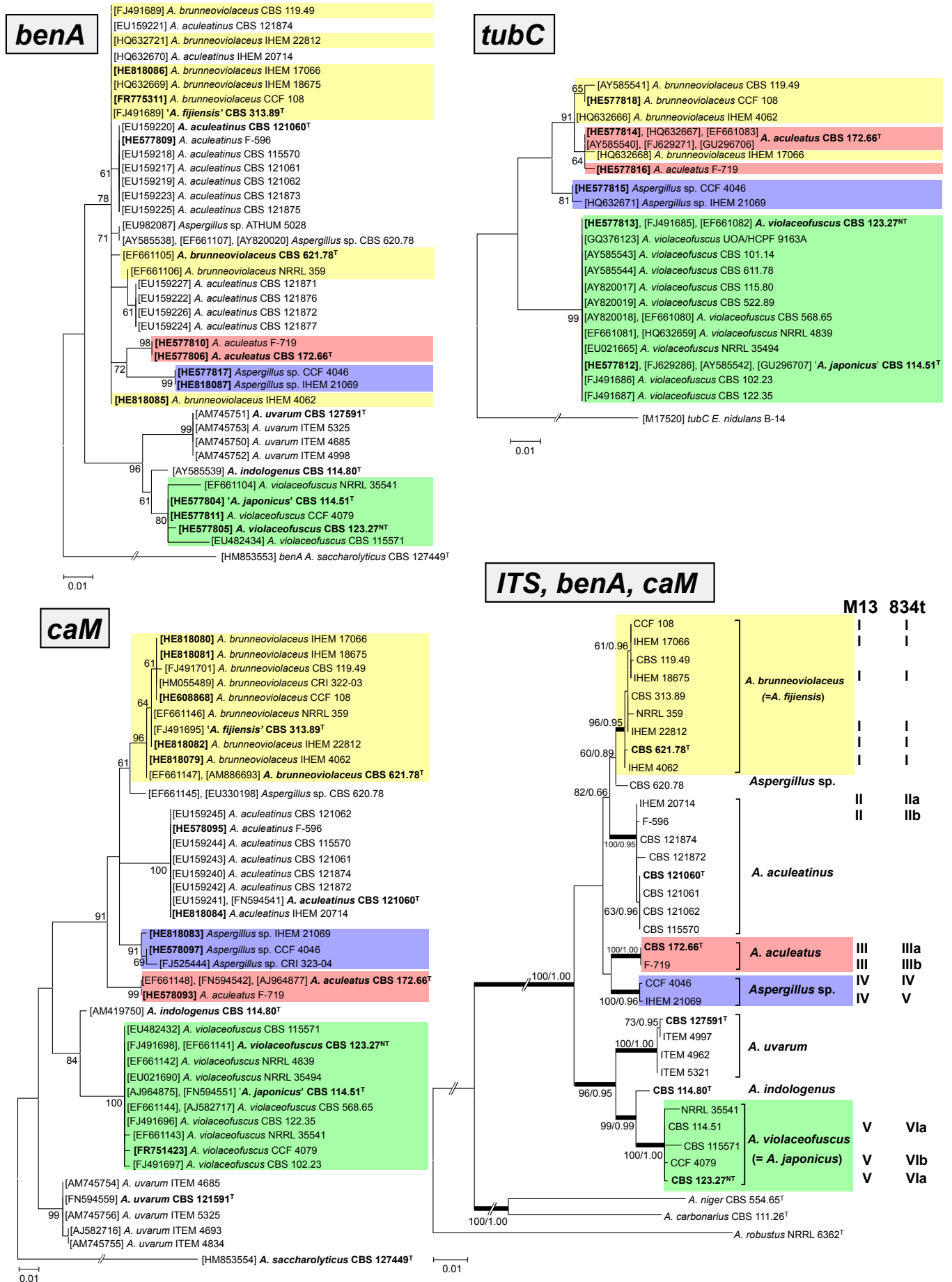
Fig. 3 The ML trees with the highest log likelihood score based on partial *benA* (top left), *tubC* (top right) and *caM* (bottom left) gene sequences. A tree combining the ITS region, partial *benA* and *caM* gene is shown on the bottom right. Only bootstrap values > 60 % are shown. In the combined tree, Bayesian posterior probabilities are indicated as the second value above the nodes. Branches supported by a bootstrap value greater than 95 % and 0.95 pp. are thickened. The names of the type isolates and accession numbers of sequences deposited in this study are in **bold** print. The results of fingerprinting methods are listed in the combined tree.

Thermal cycling parameters were initial denaturing for 2 min at 96 °C, followed by 40 cycles of 30 s at 96 °C, 45 s at 44 °C, and 90 s at 72 °C, with a final elongation at 72 °C for 10 min. The amplified products were subjected to electrophoresis on 1.8 % agarose gels stained with ethidium bromide, and the banding patterns were visualised under ultraviolet light. The bands were scored as either present or absent for each strain. If more than two thirds of bands were shared between isolates, the patterns were evaluated as the same type and designated by the Roman numerals. If more than half and less than two thirds of bands was shared, the patterns were evaluated as subtypes and designated by lower case letter following Roman numerals of particular types.

Codon usage (CU) analysis

A dataset was used for CU analysis that involved all sequences used for construction of the phylogram in Fig. 2. The final alignment included 207 positions corresponding to 69 amino acid residues. CU characteristics were determined using program
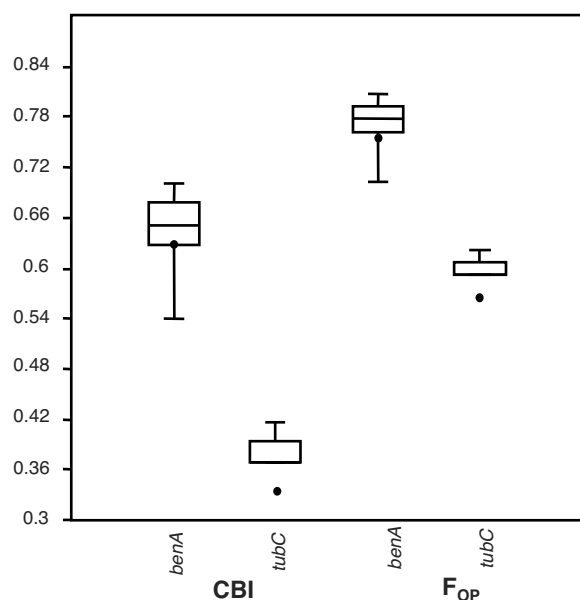
CodonW (http://codonw.sourceforge.net) (Peden 1999). The Codon Bias Index (CBI) (Bennetzen & Hall 1982) and frequency of optimal codon (F_{OP}) (Ikemura 1981) were calculated taking the optimal codons of *A. nidulans* (Lloyd & Sharp 1991) implemented in CodonW. Other characteristics calculated were the G+C composition in the third position of synonymous codons (GC_{3s}) and the composition of particular bases in the third position of synonymous codons (A_{3s}, C_{3s}, G_{3s}, T_{3s}).

Statistical analysis

Data analysis was performed with the software package PAST (Hammer et al. 2001). Data characterising CU did not show a normal distribution (based on Shapiro and Wilk's W-test), and therefore, the Mann-Whitney test (MWt) was used to test for significant differences in particular CU statistics between paralogues.

Source of isolates

The isolates used in this study were obtained from the Culture Collection of Fungi at the Department of Botany of Charles University in Prague (CCF), Czech Republic; Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands; and the Belgian Coordinated Collections of Micro-organisms (BCCM/IHEM), Brussels, Belgium. Isolates F-719 and F-596 were obtained from the Czech Collection of Microorganisms (CCM), Masaryk University, Faculty of Science, Brno, Czech Republic.

RESULTS

TubC paralogues in the GenBank/EMBL/DDBJ database

Phylogenetic analysis (Fig. 2) and CU analysis (Fig. 4) indicated that β-tubulin sequences of black aspergilli cluster in two distinct clades corresponding to *benA* and *tubC* paralogues. Fragments delimited by the *Bt2a*/*Bt2b* primer pair corresponding to the *tubC* paralogue only have two introns (the intron following the 53rd amino acid residue from the beginning of both paralogues is missing in *tubC*) compared to three intron fragments in the *benA* gene (Fig. 1) as previously recognised by Peterson (2008). The affected species were *A. aculeatus*, *A. brunneoviolaceus*, *A. fijiensis*, *A. japonicus* and *A. violaceofuscus*. In addition, we amplified the *tubC* paralogue from one species that is tentatively new (Table 1, Fig. 3) using *Bt2a*/*Bt2b* primers. The species identities of some isolates for which only the *tubC* paralogue was published were questionable due to the lack of diagnostic *caM* and *benA* sequences. These taxa were sequenced and their revised identity is presented on Fig. 2, 3 and in Table 2.

Fig. 4 Codon usage bias parameters characterising sequences of *benA* and *tubC* used in the construction of the phylogram shown in Fig. 2. The examined alignment included 207 positions corresponding to 69 amino acid residues. The *benA* gene shows a notably higher level of codon bias. Black dots indicate values of *A. nidulans* (CBI: *benA* - 0.638, *tubC* - 0.339; F_{OP}: *benA* - 0.764, *tubC* - 0.571). CBI = codon bias index; F_{OP} = frequency of optimal codons.

Table 2 Species from section *Nigri* for which a *tubC* paralogue was deposited in public sequence databases and their re-determination based on clustering with the ex-type isolates in phylogenetic analysis.

A. violaceofuscus	*benA*	EF661104; EU482434; **HE577804**[1,2]; **HE577805**[1]; **HE577811**
	tubC	AY585542[1,2]; AY585543; AY585544; AY820017; AY820018; AY820019; EF661080; EF661081; EF661082[1]; EU021665; FJ491685[1]; FJ491686; FJ491687; FJ629286[1,2]; GQ376123; GU296707[1,2]; **HE577812**[1,2]; **HE577813**[1]; HQ632659
A. aculeatus	*benA*	**HE577806**[1]; **HE577810**
	tubC	AY585540[1]; EF661083[1]; FJ629271[1]; GU296706[1]; **HE577814**[1]; **HE577816**; HQ632667[1]
A. brunneoviolaceus	*benA*	**EF661105**[1]; EF661106; FJ491688[1,3]; FJ491689; **FR775311**; **HE818085**; **HE818086**; HQ632669; HQ632721
	tubC	AY585541; **HE577818**; HQ632666; HQ632668
Aspergillus sp.[4]	*benA*	**HE577817**; **HE818087**
	tubC	**HE577815**; HQ632671

[1] Sequence of the ex-type or ex-neotype strain.
[2] Ex-type of *A. japonicus*.
[3] Ex-type of *A. fijiensis*.
[4] Represented by isolates CCF 4046, IHEM 21069 and CRI 323-04.
Sequences deposited in this study are in **bold** print.

Table 3   Studies that misidentified the *tubC* paralogue as the *benA* gene or referenced *tubC* sequences.

Study	Focus of interest
Ferracin et al. (2012)	Food mycology
Hendrickx et al. (2012)	Medical mycology, taxonomy
Andersen et al. (2011)	Comparative genomics
Arabatzis et al. (2011)	Medical mycology
Howard et al. (2011)	Medical mycology
Meijer et al. (2011)	Taxonomy, physiology
Silva et al. (2011)	Taxonomy
Sørensen et al. (2011)	Taxonomy
Varga et al. (2011)	Taxonomy
Noonim et al. (2008)	Taxonomy
Perrone et al. (2008)	Taxonomy
Samson et al. (2007)	Taxonomy
Varga et al. (2007)	Taxonomy
Perrone et al. (2006)	Taxonomy, food mycology
de Vries et al. (2005)	Taxonomy
Samson et al. (2004)	Taxonomy

The sequences of the *tubC* paralogue are predominant sequences among β-tubulin sequences deposited in GenBank for *A. japonicus* and *A. violaceofuscus* (*A. japonicus* is here considered as synonymous to *A. violaceofuscus* - see below). For *A. aculeatus*, there was no *benA* sequence deposited that could be assigned to this species, although the species name '*A. aculeatus*' was the most frequent name under which sequences of species from the *A. aculeatus* clade are deposited. Similarly for *A. violaceofuscus*, there were only two *benA* sequences belonging to non-type isolates; other sequences represented the *tubC* paralogue (Table 2). The absence of *benA* sequences in databases for type specimens of *A. aculeatus*, *A. japonicus* and *A. violaceofuscus* is taxonomically important. Appropriate *benA* sequences were amplified in this study (see below) and deposited in the EMBL database (Table 2).

Distribution of tubC paralogue in section Nigri

In section *Nigri*, the β-tubulin *tubC* paralogue is most likely only present in some taxa from the *A. aculeatus* clade sensu Varga et al. (2011). Concerning this clade, no *tubC* sequences were published for *A. aculeatinus*, *A. indologenus* and *A. uvarum.* PCR tests with *A. aculeatinus* F-596 and IHEM 20714, the only one of the three species mentioned and included in our study, confirmed the presence of *tubC*. The *tubC* paralogue was absent in *A. carbonarius* and *A. niger*, two taxa with complete genome sequences.

Taxonomical consequences

The sequences of the *tubC* paralogue used in combined datasets with *benA* sequences resulted in long, marginal, well-supported branches in phylogenetic trees (Samson et al. 2004, 2007, de Vries et al. 2005, Varga et al. 2007, 2011, Noonim et al. 2008, Sørensen et al. 2011). The tree topologies were clearly different from those constructed based on *caM* sequences, and the relationships between taxa were distorted. Aside from taxonomical works, the *tubC* paralogue was also amplified in studies focused on medical or food mycology (Table 3).

Our data indicate that the position of *A. japonicus* and *A. violaceofuscus* as separate taxa that was proposed by Varga et al. (2011) is not supported by sequence data and by two fingerprinting methods used (Fig. 3). There are no unique positions in alignments shared across isolates that were designated as *A. violaceofuscus* by Varga et al. (2011) differentiating them from isolates of *A. japonicus*. A previous molecular study of Peterson (2008) also indicated that a neotype isolate of *A. violaceofuscus* (CBS 123.27 = NRRL 360) clusters with *A. japonicus* isolates. Because *A. violaceofuscus* was described

earlier, *A. japonicus* should be treated as a synonym of *A. violaceofuscus*.

Based on the ITS and *benA* data, *A. fijiensis* is indistinguishable from *A. aculeatinus* and *A. brunneoviolaceus* (Fig. 3). The *caM* data separate *A. fijiensis* from *A. aculeatinus* and only one unique position in the *caM* locus segregates weakly supported clades with an ex-type isolate of *A. brunneoviolaceus* (CBS 621.78^T and IHEM 4062) and *A. fijiensis* (CBS 313.89^T, IHEM 22812 and NRRL 359) (Fig. 3). The intraspecies genetic distances between two *A. fijiensis* isolates (CBS 313.89^T and CBS 119.49) designated by Varga et al. (2011) are similar to those between ex-type isolates of *A. fijiensis* and *A. brunneoviolaceus*. Two fingerprinting methods previously used in *Aspergillus* (Nováková et al. 2012) and *Penicillium* (Tuthill 2004) for typification at species and subspecies level also showed no support for *A. fijiensis* as separate species (Fig. 3). Similarly, no unique morphological features differentiating *A. fijiensis* from *A. brunneoviolaceus* were found (Hubka, unpubl. data). Due these results, *A. fijiensis* is synonymised with *A. brunneoviolaceus.*

The isolate CCF 4046 most likely represents an undescribed uniseriate black *Aspergillus* species. Its monophyly is supported by sequence data for *benA*, *tubC*, *caM* (Fig. 3) and *rpb2* (data not shown). Isolate IHEM 21069 and probably also CRI 323-04 represent additional isolates. This species is proposed under the name *A. floridensis* as a new uniseriate species by Jurjevic et al. (unpubl. data).

Aspergillus violaceofuscus Gasperini, Atti Soc. Tosc. Sci. Nat. 8: 326. 1887.

   = *Aspergillus japonicus* Saito, Bot. Mag. (Tokyo) 20: 61. 1906.

Aspergillus brunneoviolaceus Bat. & H. Maia, Anais Soc. Biol. Pernambuco 13: 91. 1955.

   = *Aspergillus fijiensis* Varga, Frisvad & Samson, Stud. Mycol. 69: 9. 2011.

BenA amplification in Aspergillus section Nigri and primer specificity

The primer combination of *Bt2a* and *Bt2b* is the most important in amplification of the *benA* gene in *Aspergillus*. Under standard conditions (annealing 55 °C), we found that this combination shows specificity for the *tubC* paralogue or both paralogues are amplified simultaneously (Fig. 5). After the annealing temperature is increased, the specificity for the *tubC* paralogue is increased (Fig. 6).

We tested all possible combinations of some previously published primers (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997) marked on Fig. 1 and a newly designed primer *Ben2f* on a set of uniseriate as well as biseriate species from section *Nigri* (Table 4) using two different settings. The problems with primer specificity are completely solved when using the newly designed *Ben2f* primer as the forward primer (Fig. 5) in combination with *Bt2b* and *T22* reverse primers (Table 4). Another primer combination functional across species from section *Nigri* and showing good *benA* specificity was the *T10*/*T22* combination.

Divergence of β-tubulin paralogues

In *A. nidulans* (section *Nidulantes*), the amino acid sequence of *tubC* is highly divergent from the *benA* gene (16 %; resp. 15.6 % when comparing fragments bordered by *Bt2a*/*Bt2b* primers). Compared with *A. nidulans*, the divergence is much smaller in *A. aculeatus* from section *Nigri* (11.6 %; resp. 8.7 % in BT2-fragment). This lower divergence between both paralogues most likely participated in the decreased specificity of

Table 4   Selected primer combination and their specificity to β-tubulin paralogues across species belonging to *Aspergillus* section *Nigri*.

Primer combination	Bt2a/Bt2b		Bt2a/T224		Bt2a/T222		Bt2a/T22		T10/Bt2b		T10/T22		Ben2f/Bt2b		Ben2f/T224		Ben2f/T222		Ben2f/T22	
PCR conditions[1]	I	II	I	II	I	II	I	II	I	II	I[3]	II	I	II	I	II	I	II	I	II
uniseriate																				
A. violaceofuscus	t	bt	t	mp	mp	mp	t	bt	bt	bt	b	bt		b	mp	mp		b	b	b
A. aculeatus	t	bt	bt	bt	mp	b	bt	bt	bt	bt	b	bt	b	mp	b	b	b	b	b	b
A. aculeatinus	b	b	bt	b	b	b	bt	bt	bt	bt	b	b	b	b	b	mp	b	b	b	b
A. brunneoviolaceus	bt	bt	bt	b	b	b	b	bt	bt	bt	b	b	b	b	b	b	b	b	b	b
Aspergillus sp.[2]	bt	bt	bt	bt	b	b	b	bt	bt	bt	b	b	b	mp	b	mp	b	b	b	b
biseriate																				
A. carbonarius	b	b	mp	b	b	0	b	mp	b	b	b	b	b	b	b	b		0	b	b
A. niger	b	b	b	b	mp	0	b	mp	b	b	b	b	mp	mp	mp	b		0	b	b
A. tubingensis	b	b	mp	b	b	0	b	mp	b	b	b	b	b	b	b	b		0	b	b
A. piperis	b	b	mp	b	b	0	b	mp	b	b	b	mp	b	mp	mp	mp		0	b	b

[1] Two different PCR cycling conditions were used for amplification; Type I - 'standard' PCR conditions, Type II - touchdown cycling conditions; for details see Material and methods.
[2] Represented by isolates CCF 4046 and IHEM 21069.
[3] Amplification products are relatively weak, it can be improved by increasing the number of PCR cycles to 40, although *tubC* fragment can occur in *A. violaceofuscus* and *A. aculeatus*.
Amplification products: b = *benA*[1]; t = *tubC*; bt = *benA* and *tubC* fragment together; mp = multiple non-specific products; 0 = no product.

the widely used β-tubulin primer combination *Bt2a/Bt2b* (Glass & Donaldson 1995).

Codon usage

There were significant differences in codon usage bias between paralogues *benA* and *tubC*. CBI and F_{OP} statistics were significantly higher (MWt p-values $< 10^{-13}$) for the *benA* gene (Fig. 4) indicating its higher level of codon bias. Other statistics that were significantly different between paralogues comprised GC_{3s}, A_{3s}, G_{3s}, T_{3s} (all MWt p-values $< 10^{-13}$).

DISCUSSION

Paralogous genes and taxonomy

The combination of sequences belonging to paralogous genes with non-homologous functions in the same phylogenetic analysis is a great risk and might cause incongruences within and between datasets. Taxonomists prevent the impact of paralogous genes on taxonomic conclusions by using a polyphasic approach. Molecular data from several non-linked loci are combined with morphological, physiological and other traits to define interspecies boundaries (Samson & Varga 2009). Regarding quickly speciating species such as *Aspergillus* (sequence divergences between sibling species are mostly 0–5 % depending on the locus examined), the importance of morphology is often only secondary. Together with the growing number of cryptic species, taxonomy misses an important tool for elimination of the impact of paralogous genes on taxonomical conclusions.

In fungal genomes, an inconstant number of β-tubulin paralogues can be found (Hubka 2011) that can be randomly amplified when using primers with low specificity. Keeling et al. (2000) tried to construct a phylogeny of Fungi based on β-tubulin gene sequences. In several cases, the authors amplified two or three paralogues for some species using newly designed primers. The results of such an analysis and its interpretation had a very limited value. β-tubulin primers for Fungi were also designed by Einax & Voigt (2003), but their specificity is disputable due to the number of by-products on depicted electrophoretograms.

Tools for distinguishing β-tubulin paralogues

Peterson (2008) first noted differences in intron numbers between β-tubulin amplicons of uniseriate black aspergilli amplified by the *Bt2a/Bt2b* primer pair. Because of doubt about the homology, the sequences of β-tubulin genes were not used in multilocus analyses of sections *Nigri*, *Usti* and *Nidulantes*. This finding was not further kept in mind by taxonomists, and fragments with a variable intron number were combined in recent taxonomical studies (Table 3). Peterson's finding was misinterpreted by Sørensen et al. (2011) as a variation in intron number in the *benA* gene of *A. aculeatus* and *A. violaceofuscus*, compared to the other members of section *Nigri*. In fact, there is no difference in the number of introns between black aspergilli for the *benA* gene. Most of the *Aspergillus* species produce three intron *Bt2a/Bt2b benA* fragments. The homologous fragments of the *tubC* paralogue in section *Nigri* include only two introns (Fig. 1). The differences in fragment lengths can also be observed on electrophoretograms (Fig. 5, 6). Nevertheless, intron number cannot be used as reliable marker for distinguishing of both paralogues as presumed by Peterson (2008). Across *Aspergillus* species fragments can be found from *benA* as well as *tubC* with two or three introns in fragments bordered by the *Bt2a* and *Bt2b* primers (Hubka & Kolařík, unpubl. data).

The primer pair *Bt2a/Bt2b* shows excellent usability across Fungi. Nevertheless, both primers were designed based on a small number of *benA* sequences (Glass & Donaldson 1995), and this broad usability could be accompanied with insufficient
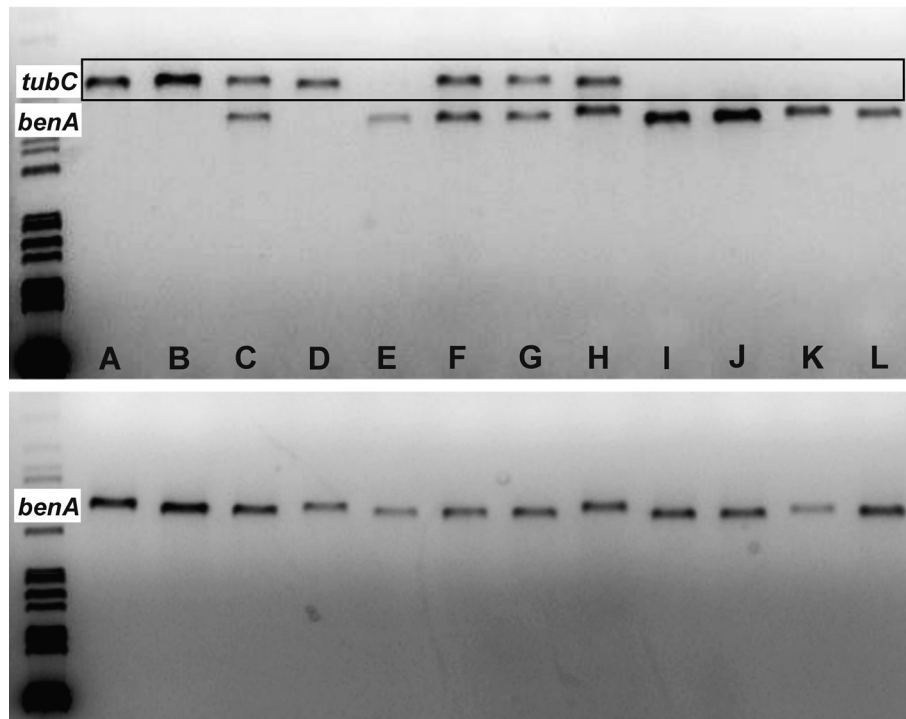
Fig. 5 The electrophoretogram showing amplification products of the β-tubulin gene. The PCR reaction was performed at an annealing temperature of 55 °C. The reaction with primers *Bt2a* and *Bt2b* is shown on the upper part of the image. The lower part shows a reaction with primers *Ben2f* and *Bt2b*. The taxa used for primer testing are listed in Table 1 and designated A-L. The specificity of the *Bt2a* and *Bt2b* primer pair is apparently low in contrast to primer pair *Ben2f* and *Bt2b* that is highly *benA* specific.

Fig. 6 The electrophoretogram showing the behaviour of primer pair *Bt2a* and *Bt2b* in a temperature gradient (annealing 50–60 °C). The specificity for the *tubC* paralogue increases with increasing annealing temperature. *Aspergillus aculeatus* CBS 172.66[T], *A. violaceofuscus* CBS 114.51 (the ex-type of *A. japonicus*) and *A. violaceofuscus* CBS 123.27[NT] were used for testing.

specificity in some taxa. Decreased divergence between paralogues in taxonomically important regions bordered by *Bt2a/Bt2b* primers in section *Nigri* most likely participated in altered specificity of the *Bt2a/Bt2b* pair that preferentially amplifies *tubC* paralogues or both paralogues at the same time. We solved this problem with non-specifics primers in section *Nigri* by implementation of a new *Ben2f* primer that showed good *benA* specificity and wide usability across black aspergilli (Fig. 5, Table 4). Further studies are needed to verify the specificity of β-tubulin primers in other sections of the genus *Aspergillus* and also in other fungi. We can also hypothesise that taxonomy of other fungal groups may be affected by illegitimate use of paralogous genes analogically to black aspergilli. The GenBank database includes more than 25 000 β-tubulin sequences, and we only examined a subtle fraction.

High divergence of β-tubulin paralogues was first observed in *A. nidulans* by May et al. (1987). This divergence is accompanied by a different codon spectrum used by paralogues. The *benA* gene is highly biased and its codon spectrum is markedly limited. In contrast, no preferences in the use of synonymous codons can be observed in *tubC*. As we show here, this different

level of codon bias can be used as an excellent marker for distinguishing *benA* and *tubC*. CBI and F_{OP} statistics characterise the level of codon usage bias by one value and are suitable for gene comparison (Fig. 4). In addition, optimisation of both characteristics is available for *A. nidulans* (Peden 1999). F_{OP} is a simple ratio between the frequency of optimal codons (that appear to be translationally optimal) and the total number of synonymous codons. It ranges from 0 to 1 (when a gene is entirely composed of optimal codons). CBI is a measure of codon bias towards a subset of optimal codons and is similar to F_{OP}. In a gene with extreme codon bias, CBI may equal 1.

Codon usage bias also has important functional consequences. Highly biased genes are generally highly expressed and perform important functions in contrast to genes with low levels of codon bias (Sharp et al. 1986, Sharp & Devine 1989). It was also demonstrated that genes with similar codon usage are usually co-expressed during the life cycle (Lavner & Kotlar 2005, Najafabadi et al. 2009). This is in agreement with previous observations regarding functions of β-tubulin species. Products of the *benA* gene play an important role during whole vegetative growth: they participate in the formation of the mitotic spindle

and in movement of organelles including the nucleus (Oakley & Morris 1980, 1981). The product of the *tubC* paralogue participates in conidiogenesis in *A. nidulans* but is not essential for this process (May et al. 1985, Weatherbee et al. 1985, May 1989). Further studies are needed to uncover if notably different divergence between paralogues in *A. nidulans* and in black aspergilli also has functional consequences.

Searching for sequence similarity via Blast (http://blast.ncbi. nlm.nih.gov/Blast.cgi) offers a very simple method for discrimination between both paralogues. Annotated sequences labelled as *benA* or *tubC* that were amplified in this study are available under the accession numbers listed in Table 1 and 2. The divergence between *benA* and *tubC* is sufficiently high for their clear differentiation.

Taxonomic notes

Aspergillus aculeatus and ˈ*A. japonicus*ˈ are well-supported species based on molecular data (Pařenicová et al. 2001, Samson et al. 2007), although both species are indistinguishable based on morphology (Hamari et al. 1997). In the past, *A. violaceofuscus* was treated as a colour variant and synonym of *A. aculeatus* by Raper & Fennell (1965). Varga et al. (2011) treated *A. violaceofuscus* as a valid species related to *A. japonicus* based on a polyphasic approach, although the analysis of the β-tubulin locus was based on a mixed *benA*/*tubC* dataset. We re-examined the *benA*, *tubC* and *caM* sequences of isolates treated as *A. violaceofuscus* and *A. japonicus* by Varga et al. (2011), and we did not observe any molecular support for separation of *A. japonicus* and *A. violaceofuscus*. Although differences in conidial shape can be observed in the neotype culture of *A. violaceofuscus* CBS 123.27 and the ex-type culture of *A. japonicus* (CBS 114.51), unique DNA characters at multiple loci should be present as a gold standard for *Aspergillus* species delimitation (Samson & Varga 2009). Additionally, the ex-type cultures share the same banding patterns provided by fingerprinting methods (Fig. 3).

Aspergillus brunneoviolaceus (CBS 621.78 = NRRL 4912), described by Batista & Maia (1955), was treated as a synonym of *A. japonicus* by Raper & Fennell (1965). We examined sequence data provided by Peterson (2008) that indicated that *A. brunneoviolaceus* should be a valid species. This taxon was omitted by Varga et al. (2011), and two very closely related isolates were proposed as a new species; *A. fijiensis*, although there is very low phylogenetic support for its delimitation (Fig. 3) from *A. brunneoviolaceus*. The extrolite data do not clearly support *A. fijiensis* as a separated species. Secondary metabolites produced by *A. fijiensis* differ among isolates CBS 119.49 and 313.89 that were used for description (Pařenicová et al. 2001, Varga et al. 2011). Additional differences are found in an ex-type isolate of *A. brunneoviolaceus* (Pařenicová et al. 2001). Consequently, *A. fijiensis* is here treated as synonymous to *A. brunneoviolaceus*. The position of another related isolate CBS 620.78 remains unresolved (Fig. 3).

Although these taxonomic remarks are not in direct consequence with illegitimate use of the *tubC* paralogue, confusion associated with *tubC* was introduced in *Aspergillus* section *Nigri* taxonomy and complicates description of new uniseriate taxa. There is a substantial call for searching for new molecular and physiological markers that are usable in the classification of black aspergilli. The concept of several recently described species is only based on genetic differences at one locus. The morphological concept is insufficient and the extrolite data are not fully resolved due to a number of newly described or revived species in the *A. aculeatus* clade, and intraspecific differences are found depending on the isolate tested and the methodology used (Pařenicová et al. 2001, Noonim et al. 2008, Perrone et al. 2008, Sørensen et al. 2011, Varga et al. 2011).

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