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研究报告

Aspergillus germanicus, a new Chinese record of *Aspergillus* section *Usti*

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Abstract: [Objective] Identification and reporting two *Aspergillus* isolates as a new Chinese record species, i.e, *A. germanicus*. **[Methods]** Polyphasic studies using morphological characters and *clamodulin* gene, *β-tubulin* gene and rDNA ITS1-5.8S-ITS2 sequences. **[Results]** Based on the comparisions of morphological and molecular characters of the two *Aspergillus* isolates (AS3.15303 and AS3.15304, isolated from soil in Shandong Province, China) with the ex-type of *A. germanicus* (CBS 123887), the two Chinese isolates were identified as *A. germanicus*. **[Conclusion]** We confirmed that *A. germanicus* was a new Chinese record of *Aspergillus* section *Usti*.

Keywords: Phylogenetics, Soil fungi, Taxonomy

日耳曼曲霉(*Aspergillus germanicus*)—曲霉属焦曲霉组一个我国新记录种

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摘 要: 【目的】鉴定并报道我国一个曲霉新纪录种，即日耳曼曲霉。【方法】采用形态学性状及钙调蛋白和β-微管蛋白基因部分序列及核糖体 DNA ITS1-5.8S-ITS2 序列进行多相系统分类学分析。【结果】根据与日耳曼曲霉 *Aspergillus germanicus* 模式菌株 CBS 123887 的形态学和分子性状的比较分析，两株分离自中国山东泰山地区土壤的曲霉菌(AS3.15303 和 AS3.15304)被鉴定为日耳曼曲霉 *A. germanicus*。【结论】参考我国迄今已报道的曲霉物种，确定日耳曼曲霉 *A. germanicus* 是曲霉属焦曲霉组的一个我国新记录种。

关键词: 种系学，土壤真菌，分类学

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1 Introduction

Aspergillus ustus Group was formerly established as one of the 18 Groups under the genus *Aspergillus* according to Raper and Fennell, which accommodated five species: *A. conjunctus* Kwon-Chung & Fennell, *A. deflectus*, *A. panamensis* Raper & Thom, *A. puniceus*, and *A. ustus*[1]. Later, Gams et al. established section *Usti* Gams et al. under Subgen. *Nidulantes* Gams et al. to include these five species[2]. But members of the *A. nidulans* Group share many characters with those of the *Aspergillus ustus* Group in bearing small vesicles, sinuous stipes, biseriate sterigmata, and globose echinulate conidia. Thus, Kozakiewicz transferred two species, *A. granulosus* and *A. pseudodeflectus* from *A. versicolor* Group to *A. ustus* Group, because they presented some similar morphological characters of *Aspergillus ustus* Group. While, *A. deflectus* was moved out of *Aspergillus ustus* Group and a new group, namely, *A. deflectus* Group was established to accommodate this species, in addition, *A. pulvinus* Kwon & Fennell and *A. silvaticus* Fennell & Raper transferred from section *Versicolores* and section *Nidulantes*, respectively, were also included in this Group[3].

The study of Klich indicated that *A. pseudodeflectus* was closely related to *A. ustus* and *A. nidulans* (Eidam) G. Winter, but supported the placement of *A. granulosus* in section *Versicolores* rather than section *Usti*[4]. By contrast, the study of Peterson based on D1 and D2 regions of the nucLSU rRNA gene showed that *A. granulosus* clustered with *A. pseudodeflectus*, *A. puniceus* and *A. ustus*, and *A. deflectus* was not closely related to these three species. His study also showed that most species in section *Nidulantes* (including *A. heterothallicus*), section *Versicolores* and section *Usti* clustered together with 75% bootstrap support. Then Peterson eliminated section *Versicolores* and section *Usti* and transferred most members of the two sections to section *Nidulantes* with the exception of *A. conjuctus* and *A. panamensis*, which were moved to section *Sparsi*[5]. Based on morphological, chemical and molecular data, Houbraken et al. revived *A. insuetus* (Bainier) Thom & Church which had been regarded as a synonym of *A. ustus* by Raper & Fennell, and included eight species in section *Usti* sensu stricto[1,6]. Peterson added seven additional species, extending this section to include fifteen species[7]. Varga et al. reported another new member, *A. calidoustus* in this section[8]. Samson et al. proposed five new taxa including *A. germanicus* to this section, and again expanded it to accommodate twenty-one taxa, but excluding *A. ochraceoroseus* and *A. versicolor*[9]. Recently, Nováková et al. reported two new members[10], and Wang described one new species in this section[11]. Until now, section *Usti* accommodated twenty-four species.

In the monograph of Qi et al., only three species of section *Usti* of *Aspergillus* were reported in China, i.e., *A. deflectus*, *A. puniceus* and *P. ustus*[12]. Whereas, little taxonomic work had carried out on this section since then, until Wang reported one additional member of this section from China, namely, *A. keveioides*[11]. In the present study, two strains, AS3.15303 and AS3.15304 of *Aspergilus* belonging to section *Usti* were isolated from soil samples from Mount Tai, Shangdong Province. Based on the morphological comparisons and molecular phylogenetic analyses, they were identified as *A. germanicus*, which is a new record of China and thus reported here, so there are five species of section *Usti* discovered in China.

2 Materials and Methods

2.1 Isolation and morphology

Soil samples were collected underneath the leaf litter from the foot of Mount Tai in Shandong Province located in the monsoon area of moderate-temperate zone of China (36°15′17″N, 117°06′15″E) on 12 July, 2011. The average altitude of that area is 134 m with an atmospheric pressure of 1 004.1 hPa; the annual average temperature is 12.8 °C, with the monthly average of −1.4 °C in January and 26.5 °C in July; the period of frozen soil is from late October to late March; the frost-free period is about 200 days from late March to late September; the annual precipitation is 700 mm. (http://www.weather. com.cn). Samples were kept in sterilized plastic bags. Dilution plates were used in the isolation of the fungi[13]. Dichloran rose bengal chlortetracycline (DRBC) agar were used as the selective medium[14]. Two strains, AS3.15303 and AS3.15304 of *Aspergillus* were obtained and deposited at the China General Microbiological Culture Collection Center (CGMCC) in the Institute of Microbiology, Chinese Academy of Sciences, Beijing.

Cultivation was conducted using the media

Czapek yeast autolysate agar (CYA), Malt Extract Agar (MEA), CYA with 20% sucrose (CY20S), yeast extract sucrose agar (YES) and 25% glycerol nitrate agar (G25N) at 25 °C for 7 days. The growth on CYA at 5 °C and 37 °C was also assessed[1,9,15]. Colour names followed Ridgway[16]. Wet mounts for morphological examinations were prepared using culture material from colonies grown on CYA at 25 °C mounted in lactophenol without dye[1]. Optical microscopic examination and photographs were performed with an Olympus BH-2 Microscope (Olympus Co. Ltd., Japan) and a Canon Digital EOS 7D camera (Canon Co. Ltd., Japan).

2.2 Phylogenetic analyses

Total genomic DNA extraction followed the method of Scott et al.[17]. For amplification of partial *β-tubulin* gene (*BenA*), we employed the primers bt2a and bt2b described by Glass and Donaldson[18], while amplifying the rDNA ITS1-5.8S-ITS2, the primers ITS5 and ITS4 of White et al. were used[19]. To retrieve the partial *calmodulin* gene (*CaM*) sequence, the following primers were utilized, cmdAD1: 5′-GCC GACTCTTTGACTGAAGAGC-3′, cmdAD2: 5′-GCC GATTCTTTGACCGAGGAAC-3′ and cmdAD3: 5′-G CCGATTCTTTGACCGAAGAAC-3′ (sense primers); cmdQ1: 5′-GCATCATGAGCTGGACGAACTC-3′ and cmdQ2: 5′-GCATCATGAGCTGGACGAATTC-3′ (antisense primers), in which there were six combinations, but the cmdAD1 & Q2 and cmdAD2 & Q1 were used first[20]. Polymerase chain reaction (PCR) was carried out in 20 μL reaction system: 2× Eco*Taq* PCR Super Mix (+dye) 8.0 μL; genomic DNA 1.0 μL; sense primer (10 μmol/L) 0.5 μL; antisense primer (10 μmol/L) 0.5 μL; ddH_2O 10.0 μL (Beijing TransGen Biotech.). DNA amplification was performed in PTC-150 thermocycler (MJ Research). The thermal cycle protocol consisted of 94 °C for 3 min; 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 45 sec, 34 cycles; 72 °C for 5 min; 15 °C for 15 min. PCR products were electrophoresed in 2.0% agarose gel with a 100 bp DNA ladder (MBI Fermentas) at 80 V for 15 min. The gel was stained in 0.5 mg/L ethidium bromide buffer for 15 min and then viewed under 254 nm UV light. The products with good results were purified and sequenced in double directions by ABI 3700 DNA analyzer (Newtsingke BioTech.). Six gene sequences were obtained and deposited at GenBank as JQ814950 to JQ814955 (Table 1).

Raw sequences were proof-read and edited manually with BioEdit 7.0.9[21]. Edited sequences were aligned using MUSCLE implemented in MEGA version 5[22]. Thirty-nine strains of eighteen species in section *Usti* (Table 1) were analyzed with *A. versicolor* as the outgroup using the Maximum-Likelihood (ML) and Neighbor-Joining (NJ) methods, respectively, with Kimura-2 model and subjected to 1 000 bootstrap replicates.

Table 1 Forty strains included in the molecular phylogenetic analyses
表 1 共有 40 株菌用于分子系统学分析

Species 物种	Strains[a] 菌株	GenBank accession numbers GenBank登录号		
		ITS1-5.8S-ITS2	*BenA*	*CaM*
Aspergillus amylovorus Panas. ex Samson	NRRL 5813 ^{T}	EF652503	EF652327	EF652415
A. calidoustus Varga, Houbraken & Samson	CBS 114380	EF591741	EF591729	EF591716
	CBS 113228	EF591739	EF591730	EF591715
	NRRL 26162	EF652452	EF652276	EF652364
	AS 3.15302	JN982696	JN982686	JN982676
A. deflectus Fennell & Raper	NRRL 2206 ^{T}	EF652437	EF652261	EF652349
A. egyptiacus Moub. & Mustafa	NRRL 5920 ^{T}	EF652504	EF652328	EF652416
A. elongatus J. N. Rai & S. C. Agarwa	NRRL 5176 ^{T}	EF652502	EF652326	EF652414

(待续)

(续表)

A. germanicus Varga, Frisvad & Samson	CBS 123887 [T]	FJ531146	FJ531172	FJ531141
	AS3.15303	JQ814954	JQ814952	JQ814950
	AS3.15304	JQ814955	JQ814953	JQ814951
A. granulosus Raper & Thom	NRRL 1932 [T]	EF652430	EF652254	EF652342
A. heterothallicus Kwon-Chung, Fennell & Raper	NRRL 5096 [T]	EF652499	EF652323	EF652411
	NRRL 5097	EF652500	EF652324	EF652412
	AS 3.15313	JN982698	JN982688	JN982678
A. insuetus (Bainier) Thom & Church	CBS 107.25 [T]	EU076356	EU076371	EU076366
	CBS 119.27	EU076355	EU076372	EU076367
	NRRL 4876	EF652481	EF652305	EF652393
	NRRL 279	EF652457	EF652281	EF652369
A. kassunensis Baghd.	NRRL 3752 [T]	EF652461	EF652285	EF652373
A. keveii Varga, Frisvad & Samson	NRRL 1974	EF652432	EF652256	EF652344
	CBS 561.65	EU076352	EU076375	EU076364
	CBS 209.92 [T]	EU076354	EU076376	EU076365
A. keveioides L. Wang	AS 3.15305 [T]	JN982704	JN982694	JN982684
A. lucknowensis J.N. Rai, J.P. Tewari & S.C. Agarwal	NRRL 3491 [T]	EF652459	EF652283	EF652371
A. ochraceoroseus Bartoli & Maggi	NRRL 28622 [T]	EF661224	EF661113	EF661137
A. pseudodeflectus Samson & Mouch.	NRRL 6135 [T]	EF652507	EF652331	EF652419
	NRRL 278	EF652456	EF652280	EF652368
	AS3.15306	JN982697	JN982687	JN982677
	AS3.15307	JN982700	JN982690	JN982680
	AS3.15308	JN982699	JN982689	JN982679
	AS3.15309	JN982701	JN982691	JN982681
	AS3.15310	JN982703	JN982693	JN982683
A. puniceus Kwon-Chung & Fennell	NRRL 5077 [T]	EF652498	EF652322	EF652410
A. subsessilis Raper & Fennell	NRRL 4095 [T]	EF652485	EF652309	EF652397
A. ustus (Bainier) Thom & Church	NRRL 275 [T]	EF652455	EF652279	EF652367
	NRRL 4991	EF652492	EF652316	EF652404
	AS3.15311	JN982695	JN982685	JN982675
	AS3.15312	JN982702	JN982692	JN982682
A. versicolor (Vuill.) Tirab.	NRRL 238 T	EF652442	EF652266	EF652354

Note: [a]: Ex-type strains are marked with "[T]".

注：[a]：模式菌株标记有"[T]".

3 Results

3.1 Description of *Aspergillus germanicus* (Figure 1)

On CYA at 25 °C after 7 days: Colonies attaining 30–32 mm in diam., low, plane, umbonate in central areas; velutinous; conidiogenesis sparse, distributed in central areas, coloured Smoke Gray to Drab Gray (R. Pl. XLVI); mycelia white; no exudates; soluble pigment moderate, Pale Green-Yellow (R. Pl. V), reverse Green-Yellow.

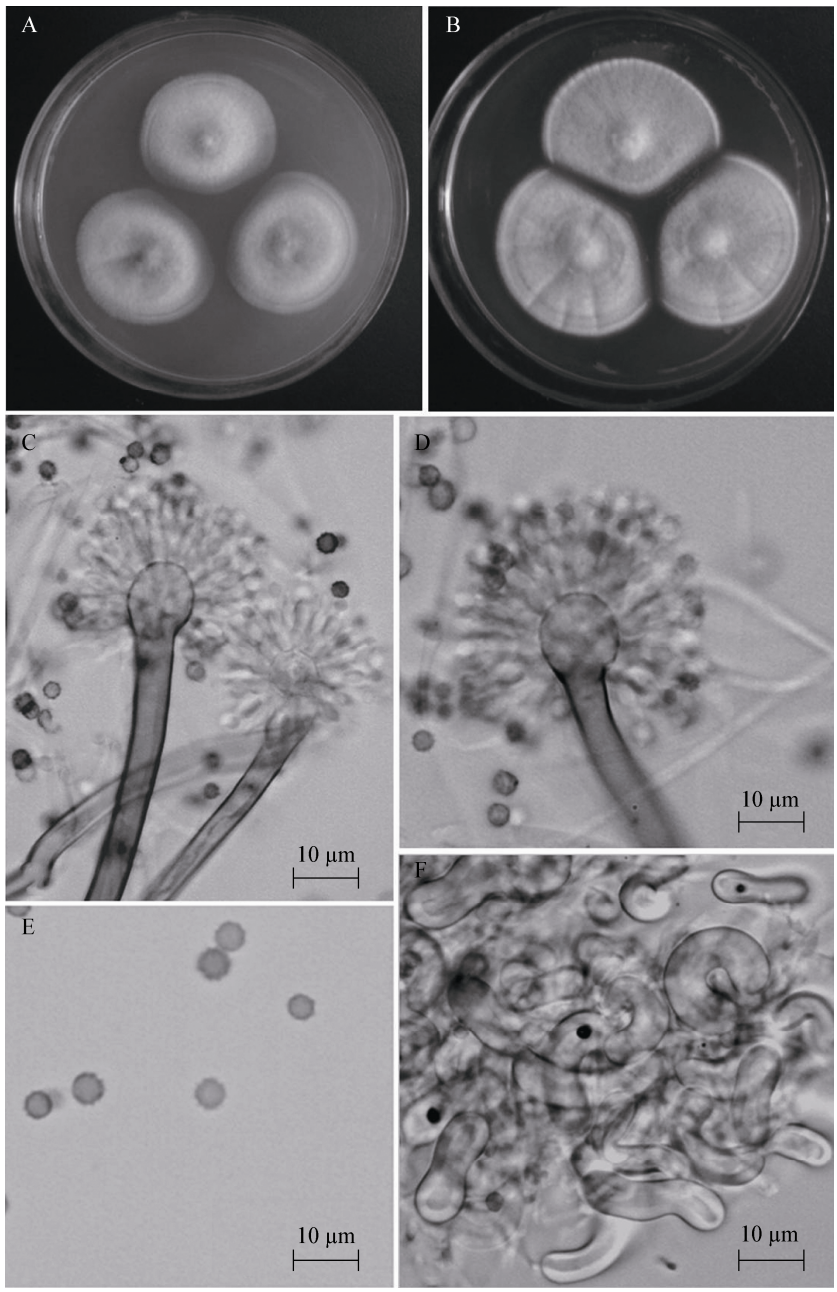

Figure 1 Morphology of *A. germanicus* AS3.15303

图 1 日耳曼曲霉 AS3.15303 的形态

Note: A–B: Colonies on CYA and YES at 25 °C after 7 days; C–D: Conidiophores on CYA; E: Conidia on CYA; F: Hülle cells on MEA.

注：A–B：在培养基 CYA 和 YES 上于 25 °C 培养 7 d 的菌落；C–D：在 CYA 上的分生孢子梗；E：在 CYA 上的分生孢子；F：在 MEA 上的壳细胞.

On Malt Extract Agar (MEA) at 25 °C after 7 days: Colonies 36–38 mm in diam., floccose; conidiogenesis sparse, distributed in centra areas, near Smoke Gray to Drab Gray (R. Pl. XLVI); mycelia white, hülle cells sparse, aggregated into conspicuous, small white masses; no exudate and soluble pigment; reverse Baryta Yellow (R. Pl. IV).

On yeast extract sucrose agar (YES) at 25 °C after 7 days: Colonies 38–40 mm in diam., low, radially and irregularly sulcate; velutinous with slightly floccose in centers; conidiogenesis moderate, Smoke Gray to Drab Gray (R. Pl. XLVI); mycelia white with Pale Viridine Yellow tint (R. Pl. V); no exudates; soluble pigment light, Pale Green-Yellow (R. Pl. V); reverse Green-Yellow.

Colonies on CYA with 20% sucrose (CY20S) at 25 °C 7 days: Colonies reaching 33–35 mm in diam., low, plane, velvety with floccose in centers; condiogenesis sparse, Smoke Grey to Drab Gray (R. Pl. XLVI); mycelia white; exudate and soluble pigment absent; reverse Green-Yellow.

On 25% glycerol nitrate agar (G25N) at 25 °C in 7 days: Colonies 21–25 mm, low, plane; velutinous; conidiogenesis sparse, Pale Vinaceous-Fawn (R. Pl. XL); no exudate and pigment; reverse light yellow.

On CYA at 37 °C in 7 days: Colonies 5–7 mm.

On CYA at 5 °C in 7 days: No growth.

Conidial heads globose, (80–)120–150 μm; conidiophores arising from substratum and surface hyphae, stipes brown-coloured, heavy-walled, 180–240(–360)×(5–8) μm; vesicles ellipsoidal to spathulate, light-brown, thin-walled, (13–16)×(9–11) μm, fertile over the most parts of vesicles; biseriate, metulae (3.5–5.0)×(2–3) μm; phialides ampuliform, 5.5–7.0 μm, with short collula; conidia globose 3–4 μm, echinulate, brown-coloured in mass; hülle cells irregularly elongate, thick-walled, commonly (10–)20–30(–40)×(5–8) μm.

Isolates examined: AS 3.15303 and AS 3.15304.

Sustratum: Soil. China, Shandong Province, foot of Mount Tai, from soil, 12 July 2011, collected by Yong-Hong Zhang.

3.2 PCR amplicons and Phylogenies (Figures 2–3)

The PCR amplification of *CaM*, *BenA* and ITS1-5.8S-ITS2 regions yielded ca. 700, 400 and 600 bp replicons, respectively. The primers for *CaM* generated nearly the full length of *CaM* gene sequence, namely, from the 2nd nucleotide of the codon for the 9th amino acid Gln (Q) to the 3rd nucleotide of the codon for the 140th amino acid Asn (N), and the trimmed alignments of the three gene sequences were respectively 501, 462 and 558 characters with gaps. Figures 2–3 are the ML phylograms based on partial *CaM* and *BenA*, respectively. The NJ trees resulted from the three genes are not shown.

In the phylogenetic trees inferred from ITS1-5.8S-ITS2 data, *A. germanicus* and *A. insuetus* and *A. keveioides* were not discriminated using both ML and NJ methods (not shown). Whereas, the trees yielded from partial *CaM* and *BenA* data indicated that the two Chinese isolates with the ex-type of *A. germanicus* CBS 123887 formed one single clade with 99%–100% bootstrap support according to both ML and NJ methods. Moreover, the ML and NJ phylogenetic trees resulted from *CaM* sequences also showed that *A. germanicus* was related to *A. insuetus*, *A. keveii*, *A. keveioides* (e. g., Figure 2 of the ML tree), but the ML and NJ trees generated by *BenA* sequences both indicated that *A. germanicus* was most related to *A. keveii* (e. g., Figure 3 of the ML tree).

4 Discussion

We constructed phylograms respectively based on the individual gene instead of using the combined sequences of the three genes, because according to the concept of Genealogical Concordance Phylogenetic Species (GCPSR), when different gene trees are concordant in topology, the concordant branches represent species[23]. In this study, the two phylograms inferred from *CaM* and *BenA* both indicated that our two isolates together with the ex-type of *A. germanicus* formed one single clade with 100% and 99% bootstrap supports, respectively, which confirmed the correct identification of our two isolates (Figures 1–2). Although the phylogram resulted from ITS1-5.8S-ITS2 could not distinguish *A. germanicus* from *A. insuetus* and *A. keveioides*, yet the branch containing the above three species and *A. keveii* had no bootstrap support, which meant that this branch could be polyphyletic. Samson et al. argued the less variation of ITS1-5.8S-ITS2 in the phylogenetic analyses of aspergilli, they also discussed *CaM*, *BenA*

and *RPB2* genes, and recommended *CaM* as the supplementary genetic marker in *Aspergillus* phylogenetics[24].

Samson et al. proposed the species *A. germanicus* based on only one isolate, namely, the ex-type CBS 123887[9]. The poor sporulation, yellow reverse, spathulate vesicles, smooth-walled conidiophores, and globose echinulate conidia are the striking similar characters as those of our two isolates, though there are some subtle differences between the ex-type and our isolates. For example, the ex-type grows more slowly on CYA (about 22–26 mm in diam. after 7 days at 25 °C) than ours (about 30–32 mm in diam. after 7 days at 25 °C), and the vesicles of the ex-type are smaller which are about 14–22 mm in diam. than those of our isolates which are about 30–32 mm in diam. But these differences only indicate the variation among different strains, the above morphological identity and the molecular evidence (Figures 2–3) both verified the identification of our two isolates as *A. germanicus*.

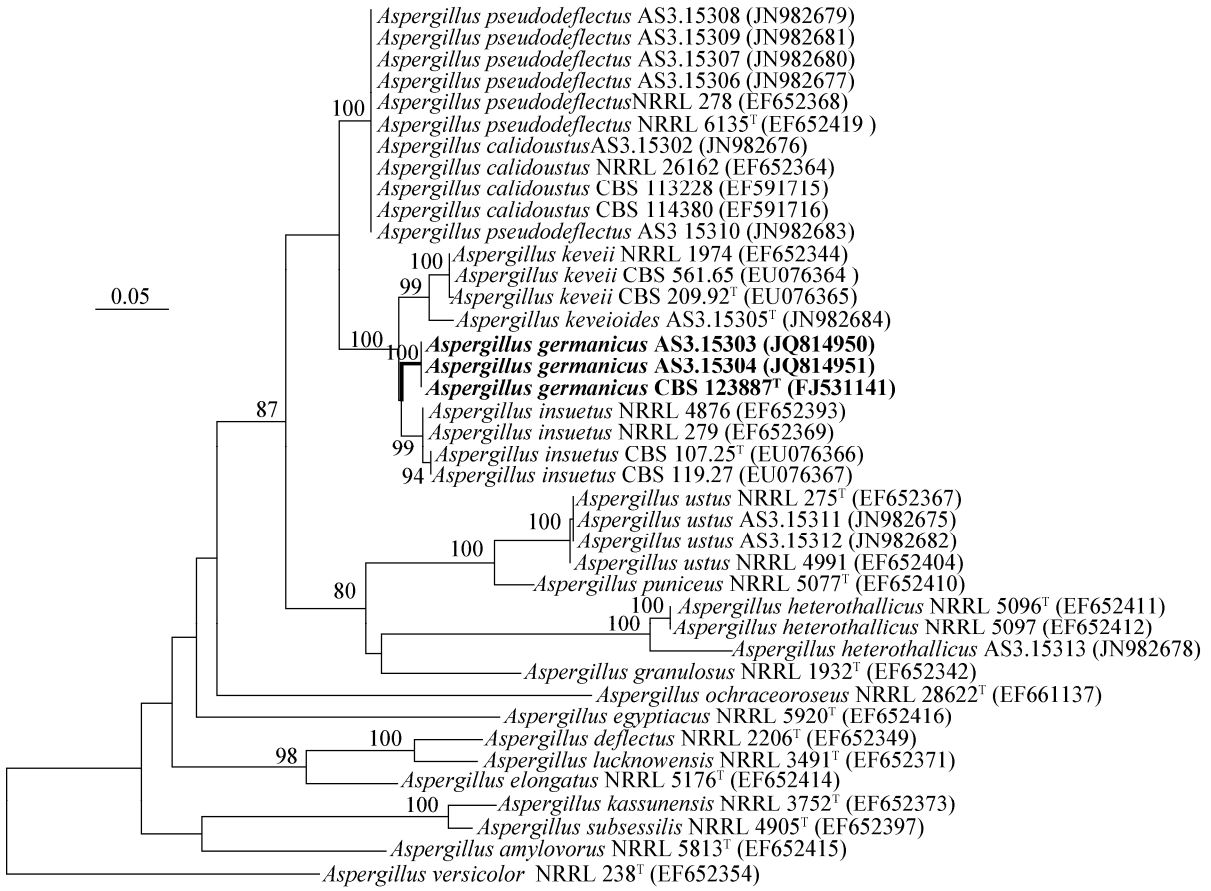

Figure 2 The ML phylogram yielded from partial *CaM* sequence data set

图 2 基于钙调蛋白基因部分序列用最大似然法推导出的系统发育树

Note: The bootstrap percentages over 70% derived from 1 000 replicates are indicated at the nodes. Bar=0.05 substitutions per nucleotide position. Ex-type strains are marked with "T". GenBank accession numbers are in parentheses. *Aspergillus germanicus* is in bold-face type.

注：用自展法进行 1 000 次重复取样得到的各分支自展支持率大于等于 70%的标注在分支节点处；标尺=0.05 个替代每核苷酸位点；模式菌株标记有"T"；GenBank 登录号在圆括号中；日耳曼曲霉用粗体标出.

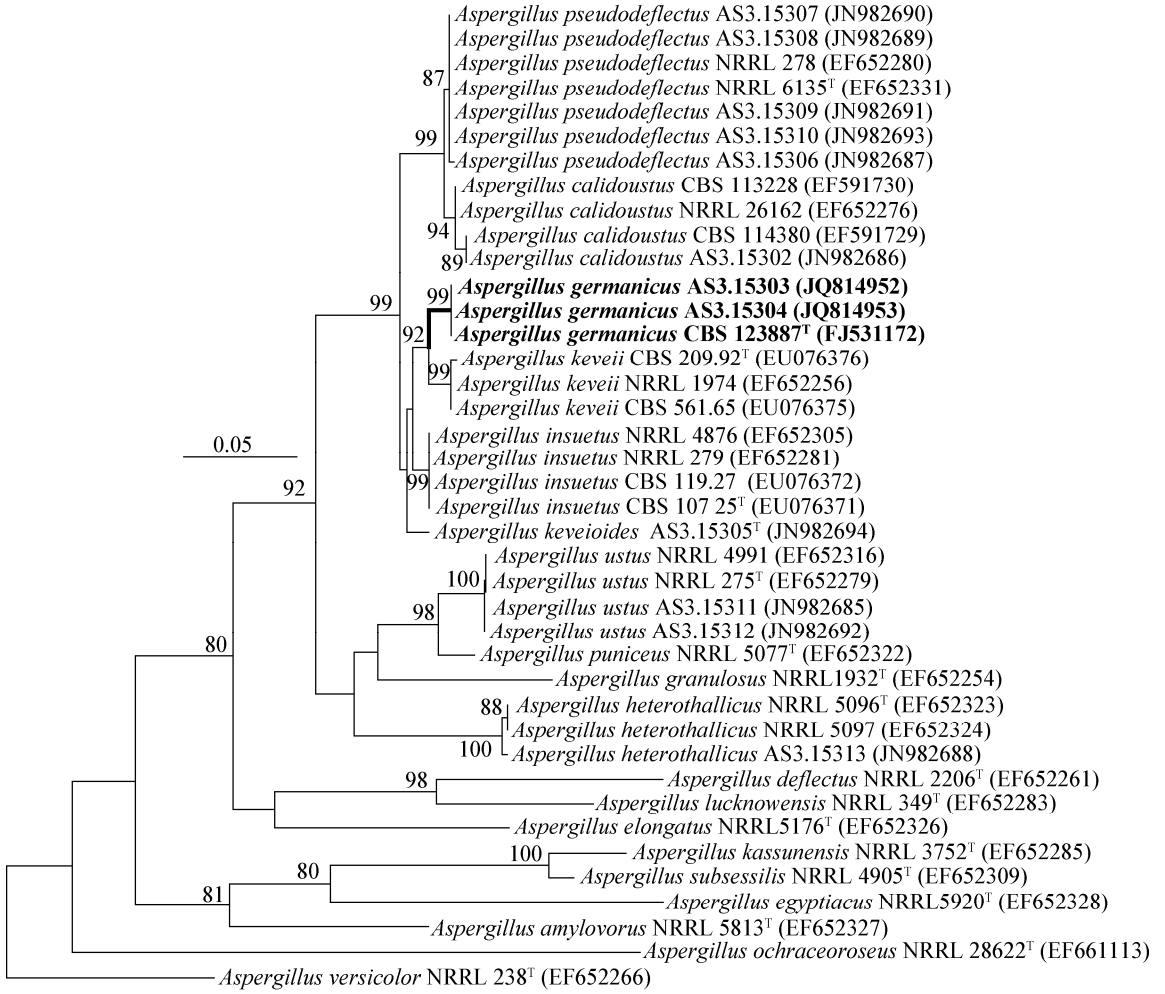

Figure 3 The ML phylogram yielded from partial *BenA* sequence data set

图 3 基于 β-微管蛋白基因部分序列用最大似然法推导出的系统发育树

Note: The bootstrap percentages over 70% derived from 1 000 replicates are indicated at the nodes. Bar=0.05 substitutions per nucleotide position. Ex-type strains are marked with "T". GenBank accession numbers are in parentheses.

注：用自展法进行 1 000 次重复取样得到的各分支自展支持率大于等于 70%的标注在分支节点处；标尺=0.05 个替代每核苷酸位点；模式菌株标记有"T"；GenBank 登录号在圆括号中.

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2015 年中国微生物学会及各专业委员会学术活动计划表(2-1)

序号	会议名称	主办/协办单位	时间	人数	地点	联系方式
1	第二届国际重症休克与脓毒症高峰论坛	中国微生物学会微生物毒素专业委员会	3 月底	1500 人	广东广州	张庆红 010-66867382 z_qinghong@aliyun.com
2	全国“发酵工程课程研讨会”	中国微生物学会生化过程模型化与控制专业委员会	4 月	120	上海	夏建业 jyxia@ecust.edu.cn
3	兽医微生物教学研讨	中国微生物学会兽医微生物学专业委员会	5 月	30	山东泰安	13683505108
4	《海洋生物高技术丛书》分册 5：海洋微生物资源开发利用审稿会	中国微生物学会海洋微生物学专业委员会	5 月	30	山东青岛	焦炳华
5	第六届传染病防控基础研究与应用技术论坛	中国微生物学会分析微生物专业委员会	6 月	300	待定	吕相征 lvxz@cma.org.cn
6	第十五届微生物学教学和科研及成果产业化研讨会	中国微生物学会农业微生物学专业委员会和普通微生物学专业委员会联合主办	7 月	200	新疆乌鲁木齐	努尔古丽·热合曼 nurgulum@163.com
7	第三届全国昆虫-微生物联合转化有机废弃物机制及资源化利用研讨会	中国微生物学会农业微生物学专业委员会	7 月	150	山东泰安	刘玉升 ysl8877@163.com
8	全国酶工程学术研讨会	酶工程专业委员会	7-8 月	200	待定	
9	工业企业微生物安全控制技术与实践研讨会	中国微生物学会工业微生物学专业委员会	8 月	150	北京	010-53218310
10	第 12 届全国海洋药物论坛	中国微生物学会海洋微生物学专业委员会	8 月	200	浙江舟山	林文瀚 13701285168
11	第 7 届全国微生物资源学术暨国际微生物系统与分类学研讨会	中国微生物学会微生物资源专业委员会	8 月 25-30 日	400	浙江杭州	阮志勇 010-82108651-620 许学伟 0571-81963208