Characterization of *Metarhizium viride* Mycosis in Veiled Chameleons (*Chamaeleo calyptratus*), Panther Chameleons (*Furcifer pardalis*), and Inland Bearded Dragons (*Pogona vitticeps*)

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ABSTRACT *Metarhizium viride* has been associated with fatal systemic mycoses in chameleons, but subsequent data on mycoses caused by this fungus in reptiles are lacking. The aim of this investigation was therefore to obtain information on the presence of *M. viride* in reptiles kept as pets in captivity and its association with clinical signs and pathological findings as well as improvement of diagnostic procedures. Beside 18S ribosomal DNA (rDNA) (small subunit [SSU]) and internal transcribed spacer region 1 (ITS-1), a fragment of the large subunit (LSU) of 28S rDNA, including domain 1 (D1) and D2, was sequenced for the identification of the fungus and phylogenetic analysis. Cultural isolation and histopathological examinations as well as the pattern of antifungal drug resistance, determined by using agar diffusion testing, were additionally used for comparison of the isolates. In total, 20 isolates from eight inland bearded dragons (*Pogona vitticeps*), six veiled chameleons (*Chamaeleo calyptratus*), and six panther chameleons (*Furcifer pardalis*) were examined. Most of the lizards suffered from fungal glossitis, stomatitis, and pharyngitis or died due to visceral mycosis. Treatment with different antifungal drugs according to resistance patterns in all three different lizard species was unsuccessful. Sequence analysis resulted in four different genotypes of *M. viride* based on differences in the LSU fragment, whereas the SSU and ITS-1 were identical in all isolates. Sequence analysis of the SSU fragment revealed the first presentation of a valid large fragment of the SSU of *M. viride*. According to statistical analysis, genotypes did not correlate with differences in pathogenicity, antifungal susceptibility, or species specificity.

KEYWORDS Clavicipitaceae, Hypocreales, granulomatous glossitis, lizards, reptiles, ribosomal DNA, visceral mycosis

Numbers of fungal infections caused by different emerging obligate and facultative pathogenic fungi in captive as well as in free-living reptiles have been increasing during the last decade (1, 2). Some of the most common reported fungal diseases in reptiles are yellow fungus disease in lizards and snake fungal disease in snakes, caused by keratinophilic ascomycetous fungi of the family Onygenaceae (Eurotiomycetes: Onygenales), identified as a member of the *Chrysosporium* anamorph of the *Nannizzia-opsis vriesii* complex (CANV) (3–6). Different reports in recent years have indicated that CANV is a reason for often fatal superficial or deep dermatomycosis in various lizard species, including veiled chameleons (*Chamaeleo calyptratus*) and inland bearded dragons (*Pogona vitticeps*) (7–12). Beside skin lesions, there have been rare cases of systemic infection (10). Another primary pathogenic species of the Onygenaceae family is *Ophidiomyces ophiodiicola*, causing snake fungal disease, a well-known disease in

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FIG 1 Tongue of veiled chameleon (*Chamaeleo calytratus*) 64996 presenting severe, diffuse, proliferative yellow-white foci ranging from 1 mm to 5 mm in diameter, caused by *Metarhizium viride* infection.

captive snakes all over the world; it is also emerging in free-ranging snake populations across the United States (1, 13–18). *Metarhizium granulomatis* is a common fungal pathogen in captive veiled chameleons kept as pets as well as in zoological enclosures; it causes dermatitis and fatal granulomatous glossitis in conjunction with systemic visceral mycosis and is therefore an important differential causative pathogen for fungal dermatitis in these species (19, 20). *Metarhizium viride* is phylogenetically closely related to *M. granulomatis*; clinical and pathological findings, such as granulomatous glossitis and visceral mycosis, are very similar (19, 21, 22). Besides one disease outbreak caused by this fungal pathogen in 4 of 50 carpet chameleons (*Furcifer lateralis*) in 1964, only four disease outbreaks in 3 panther chameleons (*Furcifer pardalis*) and 1 carpet chameleon have been described so far (21, 22). Both species are ascomycetous, filamentous fungi of the family Clavicipitaceae of the order Hypocreales (23). Another clavicipitalean entomopathogenic reptile-associated fungal pathogen is *Metarhizium anisopliae* in American alligators (*Alligator mississippiensis*) with fatal pulmonary infection and in rare cases of sclerokeratitis in humans (24, 25). *Beauveria bassiana*, *Lecanicillium* sp. (Cordycipitaceae), and *Purpureocillium lilacinum* (Ophiocordycipitaceae) are nonclavicipitaceous endophytes that cause fungal dermatitis as well as visceral mycosis in reptiles and, in the case of *P. lilacinum*, in dogs, horses, and humans as well (26–32). Nonclavicipitaceous endophytes and *M. anisopliae* are ubiquitous, filamentous, and common soil and entomopathogenic saprophytes that are used as agents to control agricultural insect pests, including nematodes or cotton aphids (33, 34).

Identification of the most fatal disease-inducing fungal species is of great importance to initiate targeted antifungal therapy and reduce mortality in reptiles. Next-generation sequencing is considered the gold standard for the accurate identification of human-pathogenic yeasts and filamentous fungi, although it is expensive and tedious (35). Phylogenetic studies of fungal pathogens of the order Hypocreales used fragments of the small subunit (SSU) of ribosomal DNA (rDNA) and DNA sequencing of the nuclear ribosome regions internal transcribed spacer region 1 (ITS-1), 5.8S, and ITS-2; domain 1 (D1) and D2 of the large subunit (LSU) of ribosomal DNA; as well as the BTUB, RPB1, RPB2, TEF, and actin protein-coding genes (19, 23).

However, profound peer-reviewed data on mycoses caused by *M. viride* in reptiles are still lacking. The aim of this investigation was therefore to obtain information on the presence of *M. viride* in reptiles kept as pets in captivity and its association with clinical signs, pathological findings, and antifungal susceptibility for improvement of diagnostic procedures and treatment options.

RESULTS

Clinical and pathological findings. Except for one panther chameleon and two inland bearded dragons, all lizards examined showed clinical signs in the form of anorexia and apathy (17 of 20 lizards; 85%). Body condition ranged from poor ($n = 10$), to reduced ($n = 2$), to good ($n = 8$), whereas all veiled chameleons revealed reduced to poor body condition ($P = 0.025$). Proliferative yellow-brown or white foci on the pharynx (5 of 20 lizards; 25%) or on the tongue (4 of 20 lizards; 20%) only or at both localizations (3 of 20 lizards; 15%) ranging from 1 mm up to 5 mm in diameter were evident in a total of 12 lizards (60%) (Fig. 1 and 2). The lizards most often affected were

FIG 2 Pharynx of inland bearded dragon (*Pogona vitticeps*) 64055 presenting multifocal yellow-white foci (black arrow) of 3 mm in diameter, caused by *Metarhizium viride* infection.

veiled chameleons (5 of 6 veiled chameleons; 83%) and inland bearded dragons (5 of 8 inland bearded dragons; 63%), and panther chameleons were less often affected (2 of 6 panther chameleons; 33%). Additionally, hemorrhages in the tongue were visible in two inland bearded dragons, and one veiled chameleon had lesions in the pharynx. Cytological smears of tongue and/or pharynx lesions revealed macrophages, lymphocytes, heterophils, and few to many conidia and short hyphae, so fungal glossitis, pharyngitis, or both were diagnosed. The remaining eight lizards revealed no pathological findings inside the oral cavity or on the tongue, but conidia without inflammatory cells were detectable. Necropsies revealed visceral mycosis in the form of multifocal yellow-white foci with diameters ranging from 1 mm up to 5 mm in various organs in 9 of 12 necropsied lizards (Fig. 3). The liver was affected in all of the lizards, followed by granulomas in the serosa ($n = 6$), lung ($n = 5$), small intestine ($n = 4$), kidney ($n = 4$), spleen ($n = 3$), brain ($n = 2$), heart ($n = 2$), ovary ($n = 2$), and adrenal gland and stomach ($n = 1$). Histopathology of foci revealed clusters of large macrophages to dense fibrous granulomas with central fibrin deposition and spherical to ovoid fungal elements measuring $2\ \mu\text{m}$ to $4\ \mu\text{m}$ in diameter as well as fragments of slender to irregularly swollen hyphae inside the granulomas (Fig. 4). Most of the lizards with visceral mycosis also had granulomatous glossitis or pharyngitis (seven of nine lizards with visceral mycosis). However, one veiled chameleon with granulomatous fungal glossitis revealed no visceral mycosis. Two chameleons, both with osteodystrophia fibrosa, revealed no findings indicative of systemic fungal infection or focal fungal lesions in the oral cavity (Table 1).

Isolation of *M. viride* and antifungal susceptibility. Isolation of *M. viride* was successful for tongue (7 of 7 lizards with granulomatous glossitis), pharynx (8 of 8 lizards with granulomatous pharyngitis), liver (9 of 9 lizards with granulomatous hepatitis), and lung (5 of 5 lizards with granulomatous pneumonia) in all lizards with pathological correlates of fungal infection. In the remaining six lizards without pathological findings indicative of fungal infection, *M. viride* was isolated from tongue ($n = 3$), pharynx ($n = 1$), and cloaca ($n = 2$). Colonies of *M. viride* showed very slow growth in general. The colony diameters measured between 0.5 and 0.9 cm as well as between 0.2 and 1.0 cm on potato dextrose agar (PDA) and Sabouraud chloramphenicol-

FIG 3 Situs of inland bearded dragon (*Pogona vitticeps*) 64055 presenting multifocal yellow-white foci ranging from 1 mm to 5 mm in diameter in the lung and liver, caused by *Metarhizium viride* infection.

gentamicin-agar (SAB-CHL-GEN), respectively, irrespective of cultivation temperatures of 25°C, 30°C, and 35°C after 5 days of incubation. Colony diameters measured between 0.9 cm and 3.0 cm irrespective of the cultivation agar or temperature after 10 days of incubation. At the beginning, the color of the colony was white to yellow with a slightly green center and dull, with a slightly powdery texture and a gray to brown reverse. The color changed during the time of incubation to gray-green with thin white margins and was flat, dull, and plicated, with a slightly powdery texture and a gray to dark brown reverse (Fig. 5). Microscopically, conidia of 1.7 to 2.7 μm in diameter and septated

FIG 4 Microphotograph of a tongue with a mycotic granuloma caused by *Metarhizium viride* from veiled chameleon (*Chamaeleo calyptratus*) 63880. Foci revealed a dense fibrous granuloma with central fibrin deposition. Shown is HE staining. Magnification, $\times 100$. The inlay reveals spherical to ovoid fungal elements 2 μm to 4 μm in diameter (black arrow) inside the granuloma. Shown is HE staining. Magnification, $\times 400$.

TABLE 1 *Metarhizium viride* genotypes and isolates identified by genomic analysis of the ribosomal DNA, origin of the isolates, pathological findings associated with mycosis, treatment regimens, outcomes, as well as additional pathological findings^c

Genotype, isolate, and GenBank accession no. of LSU	Isolate(s)/animal(s)	Origin	Organ(s) of isolation	Associated pathological finding(s), treatment, outcome, and/or additional disease
A, PO3, KY050783	5704/58310	Inland bearded dragon (<i>Pogona vitticeps</i>)	Pharynx	Granulomatous pharyngitis, visceral mycosis; voriconazole (Vfend; Pfizer, Sandwich, UK) at 10 mg/kg BW s.i.d. p.o. for 4 wk, followed by terbinafine hydrochloride (Lamisil; Novartis Pharma GmbH, Nürnberg, Germany) at 5 mg/kg BW s.i.d. p.o. for 10 wk in combination with fluconazole (Diflucan; Pfizer, Berlin, Germany) at 5 mg/kg BW s.i.d. p.o. for 2 wk; euthanized after 8 mo; articular gout, hyperplasia of melanophagocytes in the liver, pigment nephrosis
	P03/61197, 10219/64898, 10221/64996	Veiled chameleon (<i>Chamaeleo calytratus</i>)	Liver	Granulomatous glossitis, pharyngitis, visceral mycosis; ND; euthanized after 7 mo (animal 61197) and after 3 days (animal 64898); death after 3 days (animal 64996); hyperplasia of melanophagocytes in liver and pigment nephrosis
B, 5101, KY050784	5101 ^a /57866, 8419/62194	Inland bearded dragon	Pharynx, liver	Granulomatous glossitis, pharyngitis, visceral mycosis; voriconazole at 10 mg/kg BW s.i.d. p.o. for 10 days in combination with nystatin (Albrecht, Aulendorf, Germany) at 100,000 IU/kg BW s.i.d. p.o. (animal 57866), ND (animal 62194); discharged (animal 57866), euthanized after 3 days (animal 62194); hyperplasia of melanophagocytes in the liver and pigment nephrosis, adenovirus positive (animal 62194)
	5710/58585	Veiled chameleon	Tongue	NAF; ND; death after 13 mo; steatosis hepatitis, osteodystrophia fibrosa
	2001/52708, 3803 ^b /52385, 5922 ^b /58965, 10220/64843	Panther chameleon (<i>Furcifer pardalis</i>)	Small intestine, pharynx, cloaca, liver	NAF (animals 52708, 52385, and 58965), granulomatous glossitis, visceral mycosis (animal 64843); itraconazole (Itrafungol; Janssen Animal Health, Neuss, Germany) at 5 mg/kg BW s.i.d. p.o. for 2 wk (animal 58965), ND for all others; death after 24 h (animals 52708 and 64843), discharged (animals 52385 and 58965); osteodystrophia fibrosa, hyperplasia of melanophagocytes in liver (animal 52708), tenesmus caused by <i>Isoospora jaracimmani</i> (animal 58965), renal gout and hyperplasia of melanophagocytes in liver (animal 64843)
C, 3207, KY050785	P07/64055, P10/56022, 9624/63868	Inland bearded dragon	Liver, tongue	Granulomatous glossitis, pharyngitis, visceral mycosis (animals 64055 and 56022), visceral mycosis (animal 63868); voriconazole at 10 mg/kg BW s.i.d. p.o. for 9 wk (animal 56022), ND for others; euthanized after 2 days (animal 64055), after 9 mo (animal 56022), and after 7 days (animal 63868); hyperplasia of melanophagocytes in liver and pigment nephrosis
	P09/62688, 9323 ^a /63880	Veiled chameleon	Tongue	Granulomatous glossitis; ND (animal 62688), terbinafine hydrochloride at 5 mg/kg BW s.i.d. p.o. for 3 wk and amputation of the tongue (animal 63880); euthanized immediately (animal 62688), discharged (animal 63880); NAD
	3207 ^a /45934, 5203 ^b /58037	Panther chameleon	Pharynx, tongue	Granulomatous pharyngitis; terbinafine hydrochloride at 5 mg/kg BW s.i.d. p.o. for 4 wk (animal 45934), ND (animal 58037); euthanized after 8 wk (animal 45934), discharged (animal 58037); NAD (animal 45934), traumatic skin injury (animal 58037)
D, 2313, KY292416	2302 ^a /53278, 2313/53258	Inland bearded dragon	Pharynx, liver	Granulomatous pharyngitis (animal 53278), visceral mycosis (animal 53258); voriconazole at 10 mg/kg BW s.i.d. p.o. for 6 wk (animal 53278), ND (animal 53258); discharged (animal 53278), death after 24 h (animal 53258); hyperplasia of melanophagocytes in liver, articular gout, and pigment nephrosis

^aNo pathological examination was performed.^bNo pathological examination was performed, and there was no associated lesion in the pharynx or on the tongue.^cBW, body weight; NAF, no associated pathological findings; ND, no additional findings; p.o., orally; s.i.d., *semel in die* (once per day).

FIG 5 *Metarhizium viride* isolate 2313 on potato dextrose agar (Oxoid, Wesel, Germany) for 10 days under aerobic conditions at 30°C. Fungal colonies are gray with a thin white margin, flat, dull, and plicated, with a slightly powdery texture and velvety appearance.

hyphae with a length of 14 to 28 μm and a width of 0.8 to 1.5 μm are characteristic after 5 to 10 days of incubation at 30°C (Fig. 6).

Most of the isolates were unsusceptible to all eight antimycotic test disks used. Single susceptibilities against 25 μg fluconazole were evident for four isolates, and single susceptibilities against 20 μg amphotericin B were evident for one isolate. Multiple susceptibilities against 25 μg fluconazole and 100 IU nystatin as well as against 25 μg fluconazole, 100 IU nystatin, and 20 μg amphotericin B were seen for one isolate each.

ITS-1, LSU, and SSU gene sequencing. Species affiliation with *M. viride* was proven by sequencing of the ITS-1 gene, resulting in 100% identity to *M. viride* (GenBank accession number [HM195308.1](#) [19]) for all isolates. Sequencing of the SSU/ITS-1 fragment (GenBank accession number [KY050782](#)) resulted in an identical 1,965-bp sequence for all isolates. Analysis of the LSU fragment resulted in four different genotypes with sequence lengths of 3,921 bp (genotype A [GenBank accession number [KY050783](#)]), 3,810 bp (genotype B [GenBank accession number [KY050784](#)]), 4,368 bp (genotype C [GenBank accession number [KY050785](#)]), and 4,371 bp (genotype D [GenBank accession number [KY292416](#)]). Identities were up to 97% with a query of up to 75% for various fungal species of the family Clavicipitaceae (Fig. 7). Genotype A was obtained from veiled chameleons ($n = 3$) and one bearded dragon, genotypes B and C were obtained from all three lizard species, and genotype D was obtained from inland bearded dragons kept in one collection only (Table 1). There were differences in the lengths of the amplicons of primer pairs KL6a/KL5b and KL5a/KL2. Genotype A isolates were characterized by 500 bp and 1,800 bp, genotype B was characterized by 800 bp and 1,200 bp, and genotypes C and D were characterized by 900 bp and 1,800 bp, respectively. The length polymorphism was due mainly to various gaps. The LSU analysis obtained 10 single nucleotide polymorphisms among the four genotypes. However, 100% identity was evident between *M. viride* isolate UAMH 2994 (GenBank accession number [HM635079.1](#) [19]) and all four different genotypes in the D1/D2 fragment of the LSU of 1,242 bp (Fig. 7).

Analysis of the SSU fragment with two SSU sequences labeled as *M. viride* sequences (GenBank accession number [AB023949.1](#) [M. Endo and J. Sugiyama, unpublished data] and GenBank accession number [KT148629.1](#) [strain JAKA1] [J. Abraham, K. Khare, and A. M. Chacko, unpublished data]) revealed pairwise distance values of 0.979 and 6.655 for the SSU fragment (GenBank accession number [KY050782](#)) gained here. *M. viride* (GenBank accession number [AB023949.1](#)) was 100% identical to *M. granulomatis* genotype UAMH 11028 (GenBank accession number [HM635076.1](#)), and *M. viride* strain

FIG 6 Microphotograph of *Metarhizium viride* isolate 10221 taken under aerobic conditions at 30°C on SAB-CHL-GEN (Oxoid, Wesel, Germany) plates after 5 days. Conidia measured 1.7 to 2.7 μm in diameter, and septated hyphae presented a length of 14 to 28 μm and a width of 0.8 to 1.5 μm . Shown is DiffQuik staining.

JAKA1 (GenBank accession number [KT148629.1](#)) revealed 99% identity with a query of 97% to various *Trichoderma* spp. However, the SSU gained here presented 99% identity with a query of up to 100% to various species of the family Clavicipitaceae (Fig. 8).

Statistical analysis. Except for reduced or poor body condition, which was evident significantly more often in veiled chameleons, no statistically significant differences were detected between reptile species, gender, husbandry and feeding conditions, body condition, localization of fungal granulomas, and fungal genotypes. Inflammatory reactions in the form of fibrinous granulomas were identified irrespective of lizard species, gender, husbandry and feeding conditions, health status, or coinfections.

DISCUSSION

According to the results, *M. viride* was identified as cause of granulomatous glossitis, pharyngitis, and disseminated visceral mycosis in three different lizard species. Besides a disease outbreak in a collection of carpet chameleons in 1964, these are the first documented cases in veiled chameleons, panther chameleons, and inland bearded dragons (21). Pathomorphological findings are comparable to mycoses caused by *M. granulomatis* (19, 20). Observations of disease outbreaks caused by *M. granulomatis* in collections of veiled chameleons within a time frame of 10 months are suggestive of a primary fungal pathogen (19, 20). This is most likely also the case with *M. viride* infections in lizard species, which are described here. In one collection of inland bearded dragons, two individuals revealed visceral mycosis, and one more was infected

FIG 7 Maximum likelihood phylogeny inferred from analysis of LSU sequences of fungi of the family Clavicipitaceae (Hypocreales, Sordariomycetes), with *Beauveria bassiana*, as a member of a nonclavicipitaceous species of the order Hypocreales, used as the outgroup. The tree with the highest log likelihood ($-1,044.7358$) is shown and presents four newly generated LSU fragments (genotype A [GenBank accession number [KY050783](#)], genotype B [GenBank accession number [KY050784](#)], genotype C [GenBank accession number [KY050785](#)], and genotype D [GenBank accession number [KY292416](#)]). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths being measured as the number of substitutions per site. The analysis involved 12 nucleotide sequences. There were a total of 489 positions in the final data set. Reference sequences were taken from the GenBank database (<http://www.ncbi.nlm.nih.gov/>), and GenBank accession numbers are shown at the right.

by *M. viride* within a time frame of 1 month. This was also the case for another collection of inland bearded dragons, in which one lizard died due to disseminated visceral mycosis and another presented granulomatous pharyngitis at the same time. These inland bearded dragons were kept with deficits in husbandry and feeding conditions, which could cause immunosuppression and therefore trigger a disease outbreak. This was also the case in another inland bearded dragon with an adenoviral infection. However, in other lizards, no deficits in husbandry and feeding conditions or primary immunosuppressive disease was evident. Age-related disease outbreaks are a consis-

FIG 8 Maximum likelihood phylogeny inferred from the analysis of SSU sequences of fungi of the family Clavicipitaceae (Hypocreales, Sordariomycetes), with *Beauveria bassiana* and various *Trichoderma* spp., as members of a nonclavicipitaceous species of the order Hypocreales, used as the outgroup. The tree with the highest log likelihood (0.0000) is shown and presents a newly generated SSU fragment (GenBank accession number [KY050782](#)). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths being measured as the number of substitutions per site. The analysis involved 15 nucleotide sequences. There were a total of 1,685 positions in the final data set. Reference sequences were taken from the GenBank database (<http://www.ncbi.nlm.nih.gov/>), and GenBank accession numbers are shown at the right. Two unpublished SSU sequences from GenBank, mislabeled as *M. viride*, were identified as *M. granulomatis* (GenBank accession number [AB023949.1](#)) and *Trichoderma* sp. (GenBank accession number [KT148629.1](#)).

tent finding in both mycoses, as only adult lizards were affected (19, 20). According to these results, it can be stated that *M. viride* is a primary pathogenic fungal pathogen for various adult chameleons and inland bearded dragons, causing fungal granulomatous glossitis and pharyngitis as well as fatal disseminated visceral granulomatous mycosis. Isolation of *M. granulomatis* or associated disease outbreaks have not been described for reptiles other than veiled chameleons so far. Therefore, besides *M. granulomatis*, *M. viride* is an important fungal pathogen causing disseminated mycosis in veiled chameleons and furthermore in other chameleons and bearded dragons. Contrary to *M. granulomatis*-induced mycosis, fungal dermatitis or toe necrosis was absent in the cases of *M. viride*-induced mycosis described here (19, 20). Clinical signs are similar for both mycoses and include anorexia caused by a granulomatous reaction inside the oral cavity, emaciation as result of anorexia, and visceral granulomatous fungal disease. It can therefore be assumed that infection most likely takes place by the oral intake of the fungi, most likely with soil during the intake of food. The survival time for infected lizards is 8 to 10 months for both mycoses (19, 20).

Cytological findings from smears of the tongue and pharynx in the form of small conidia and short hyphae without inflammatory reactions or clinical findings in the form of lesions of the tongue or pharynx should lead to mycological cultivation, as *M. viride* can also be present in cases without an inflammatory reaction. It is likely that these findings represent an early stage of infection in which antimycotic treatments could result in the successful elimination of the fungal pathogen or at least in the prevention of systemic visceral spread. Susceptibility tests indicate that various antimycotics could be an option for treatment, but successful treatments in cases of disease outbreaks have not been achieved yet. In conclusion, *M. viride* mycosis is an important differential diagnosis in cases of visceral disseminated mycosis in lizards and especially in veiled chameleons. Furthermore, other fungi of the order Hypocreales as well as *Nannizziopsis guarroi* can be the cause of visceral mycosis in reptiles, so accurate isolation and differentiation are necessary (10, 32).

Culture requirements for *M. viride* are ordinary and the same as those described previously for *M. granulomatis* (19). Contrary to previous descriptions of culturing requirements, *M. viride* grew well at a culturing temperature of 35°C. Colony morphology is comparable to that described previously (19). In contrast to previous descriptions, smaller conidia as well as small septated hyphae were detected after 10 days of cultivation. However, mycological findings should be based on culture results, differentiation of the isolates with molecular biological methods, as well as pathomorphological correlates (2, 22). Two unpublished SSU sequences from GenBank, mislabeled as *M. viride*, were identified as *M. granulomatis* and *Trichoderma* sp., so a large fragment of the SSU from *M. viride* along with four different large fragments of the LSU are presented for the first time for upcoming continuative phylogenetic studies of fungal isolates of the order Hypocreales of the family Clavicipitaceae. Interestingly, LSU sequence analysis yielded four different genotypes, which did not correlate with differences in pathogenicity, susceptibility, or species specificity. However, further genomic analyses will be useful for the definition of genus and species boundaries for *Metarhizium* and related reptile-associated pathogenic fungi (23).

MATERIALS AND METHODS

Animals and sampling sites. Isolates of *M. viride* were obtained from a total of 20 lizards of three different species, including inland bearded dragons, veiled chameleons, and panther chameleons. All lizards were adults of both genders (12 males and 8 females) and between 1 and 7 years old. Five of the eight inland bearded dragons were obtained from two collections and presented in a time span of 1 month. All others were kept as single individuals in different households. The lizards had different clinical signs and presented at the clinic for diagnostics and treatment. The owners agreed to complete a detailed questionnaire. Based on this information, husbandry quality was classified into two categories: unremarkable (all climatic, feeding, and technical parameters were within the required ranges) and remarkable (deficits). All lizards were captive bred in Germany and had been kept for more than 4 months by the owners. Husbandry quality was classified as being unremarkable for a total of 12 lizards and remarkable for 5 inland bearded dragons from two collections and 3 panther chameleons. Remarkable husbandry conditions included the lack of a supply of minerals and UVB light and inadequate airflow. A thorough clinical examination was conducted on each individual, comprising determination of

TABLE 2 Primers used for amplification and sequencing of the SSU/ITS-1 fragment and LSU fragment of *Metarhizium viride*^a

Target	Forward/reverse primer (sequence)	Annealing temp (°C)	Amplicon size (bp) ^a	Reference
SSU	SM1 (5'-ATC TGG TTG ATC CTG CCA GTA GTC-3')/SM2 (5'-CAA TAC GCC TGC TTT GAA CAC TC-3')	62	800	37
	SM3 (5'-CTG AGA AAC GGC TAC CAC ATC C-3')/SM4 (5'-CTT CGA TCC CCT AAC TTT CGT TC-3')	62	600	
	SM5 (5'-AAC TAC TGC GAA AGC ATT TGC C-3')/SM6 (5'-CAC CTA CGG AAA CCT TGT TAC GAC-3')	61	850	
	BIG1 (5'-AGT GAA ACT GCG AAT GGC TC-3')/SM6	61	1,600	
ITS-1/5.8S rDNA	ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3')/ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3')	52	260	38
D1/D2 LSU	KL1 (5'-GCT GCG TTC TTC ATC GAT GC-3')/KL3 (5'-CCA CCA AGA TCT GCA CTA G-3')	58	1,200	39
	KL4 (5'-AGC AGG ACG GTG GTC ATG-3')/KL6b (5'-CCC TCA GAG CCA ATC C-3')	62	700	
LSU	KL6a (5'-GGA TTG GCT CTG AGG G-3')/KL5b (5'-GTC AAG CTC AAC AGG GTC-3')	52	500 ^b /800 ^c /900 ^d	
	KL5a (5'-GAC CCT GTT GAG CTT GAC-3')/KL2 (5'-ACT TAG AGG CGT TCA GTC-3')	52	1,200 ^c /1,800 ^{a,d}	

^aReferring to *Metarhizium viride*.^b*Metarhizium viride* genotype A.^c*Metarhizium viride* genotype B.^d*Metarhizium viride* genotypes C and D.^eNote the variable lengths of the amplicons of primer pairs KL6a/KL5b and KL5a/KL2, which resulted in the definition of four genotypes.

body mass and body and overall condition and inspection of the oral cavity, including the tongue. Antifungal treatment was administered to lizards, which were considered to be infected with *M. viride* according to positive results of one of the examination methods described below (i.e., cytology, histopathology, fungal culture, and molecular detection). Three of the treated animals were euthanized because of worsening of clinical signs. In the remaining four lizards, no regression of the granulomas in the pharynx could be achieved, and isolation of *M. viride* from the granulomas was still possible after completion of the different treatment regimens (Table 1).

Individually packed sterile microbiological swabs (Applimed; Châtel-St-Denis, Freiburg, Switzerland) were taken from the tongue, oral pharynx, and cloaca of all individuals. Postmortem sampling was possible for 12 lizards, as 9 had to be euthanized due to their clinical signs and 4 died. During necropsy, sterile swabs (Applimed) were taken from the oral pharynx, tongue, lungs, intestine, and liver. Necropsies, including cytological, histopathological, parasitological, and bacteriological examinations, were performed as described previously (20). Cloacal swabs were screened via PCR for Agamid adenovirus in inland bearded dragons only (36).

Cytological and histopathological examination. Impression smears from the swabs or from inner organs in the case of dead lizards were stained with DiffQuik (Dade Behring, Marburg, Germany) and examined microscopically at a $\times 1,000$ magnification. Sections of the collected organs were fixed in 4.5% neutral buffered formalin for at least 24 h. Formalin-fixed samples were dehydrated, routinely embedded in paraffin wax, and sectioned at 4 μ m. All sections were stained with hematoxylin and eosin (HE). In cases in which fungal elements were not detectable by HE staining, a periodic acid-Schiff (PAS) reaction as well as Grocott-Gomori methenamine silver (GMS) stain were used.

Isolation, cultivation, and antifungal susceptibility. Initially, the obtained swabs were used to inoculate SAB-CHL-GEN plates (Oxoid, Wesel, Germany) as well as PDA plates (Oxoid). The agar plates were incubated aerobically at 25°C, 30°C, and 35°C for 10 days. The growth and visual appearance of the cultures were documented after 5 and 10 days. Susceptibility was screened by disk diffusion antimycotic sensitivity testing. Fungal colonies were first diluted in nutrient broth with glucose (Oxoid), the fluid was then distributed on SAB-CHL-GEN (Oxoid), and the mixture was incubated at 30°C for 120 h. The utilized test disks included 1 μ g voriconazole (catalog number CT1807B; Oxoid), 25 μ g fluconazole (CT1806B; Oxoid), 8 μ g itraconazole (Neo-Sensitabs, catalog number 81812N; Rosco Diagnostica A/S, Taastrup, Denmark), 50 μ g clotrimazole (catalog number 092815029; Liofilchem, Roseto degli Abruzzi, Italy), 5 μ g posaconazole (catalog number 020916054; Liofilchem), 100 IU nystatin (catalog number CT0073B; Oxoid), 20 μ g amphotericin B (catalog number 051916048; Liofilchem), and 30 μ g terbinafine (catalog number 87412N; Neo-Sensitabs). Zones of inhibition surrounding the disks of ≤ 30 mm were defined as unsusceptible, but in most of the resistant fungal isolates, overgrowth of the disks was seen. To enable recultivation and/or further molecular biology, all sample cultures were stored frozen in cryotubes (Roti-Store cryotubes; Carl Roth, Karlsruhe, Germany).

Molecular biological differentiation. Purification of the genomic DNA was done with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Sequencing of ribosomal DNA was performed for all 20 fungal isolates. One of them was the SSU and ITS-1, including fragments of the 5.8S ribosomal DNA gene (37, 38). The second one consisted of fragments of the LSU

(39). All amplification reaction mixtures were prepared with total volumes of 25 μ l and consisted of 250 ng template DNA, 1 $\times$ DreamTaq Green buffer (Thermo Fisher Scientific, Waltham, MA, USA), 0.4 μ M each primer, 0.2 μ M each deoxynucleoside triphosphate (dNTP), and 1.0 U of DreamTaq DNA polymerase (Thermo Fisher Scientific). Cycling was performed in a thermal cycler (peqlab Pqstar 2 $\times$ gradient; VWR) by using established PCR protocols (20). The primers, annealing temperatures, and amplicon sizes obtained are listed in Table 2. Detection was performed under UV light after electrophoretic separation of PCR products in a 1.5% agarose gel and staining with ethidium bromide.

Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 6 (40). Sequences were compared with sequences listed in the GenBank database by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) (41). Evolutionary history was inferred by using the maximum likelihood method based on the Jukes-Cantor model for LSU and SSU sequences (Fig. 7 and 8) (42). The initial tree(s) for the heuristic search was obtained automatically by applying neighbor-joining (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated by using the maximum composite likelihood (MCL) approach and then selecting the topology with the superior log likelihood value (43). Codon positions included were the first, second, third, and noncoding positions. All positions with <95% site coverage were eliminated; that is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Statistical analysis. For statistical analysis, the following categories were established: reptile species, gender, husbandry and feeding conditions, body condition, localization of fungal granulomas, and fungal genotypes. Statistical analyses were conducted with SPSS Statistics 23.0 (IBM Corporation, Somers, NY, USA). Analysis of associations between variables was performed with chi-square tests. When the expected cell values fell below 5 in a chi-square analysis, Fisher's exact tests were used. Significance was established at a *P* value of ≤ 0.05 .

Accession number(s). The GenBank accession number for the newly generated SSU/ITS-1 fragment is [KY050782](#), and those for the LSU fragments are [KY050783](#), [KY050784](#), [KY050785](#), and [KY292416](#) (Table 1).

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