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Original Article

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Characterization of *Nannizziopsis guarroi* with genomic and proteomic analysis in three lizard species

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Abstract

Fungal infections in captive as well as in free-living reptiles caused by emerging obligate pathogenic fungi appear with increasing frequency and give occasion to establish new and fast methods for routine diagnostics. The so-called yellow fungus disease is one of the most important and common fungal dermatomycoses in central bearded dragons (*Pogona vitticeps*) and green iguanas (*Iguana iguana*) and is caused by *Nannizziopsis guarroi*. The aim of this study was to prove reliability in identification of *N. guarroi* with Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in comparison to molecular biological analysis of ribosomal DNA genes. In seven lizards from three different species, including central bearded dragons, green iguanas, and a European green lizard (*Lacerta viridis*), dermatomycoses caused by *N. guarroi* were diagnosed by isolation of the fungal pathogen as well as histopathological confirmation of the granulomatous inflammatory reaction in deep skin biopsies. With this survey, we proved that MALDI-TOF MS is a diagnostic tool for accurate identification of *N. guarroi*. Besides small subunit 18S rDNA (SSU) and internal transcribed spacer (ITS)1-5.8S rDNA, a large fragment of the large subunit of the 28S rDNA (LSU), including the domain (D)1 and D2 have been sequenced, for phylogenetical analysis. Large fragment of the LSU from *N. guarroi* has been sequenced for the first time. Yellow fungus disease in a European lizard species is described for the first time to our knowledge as well, which could be of importance for free-ranging populations of European lizards.

Key words: CANV, *Lacerta viridis*, *Pogona vitticeps*, *Iguana iguana*, yellow fungus disease.

Introduction

Fungal infections caused by different emerging obligate and facultative pathogenic fungi in captive as well as in free-living reptiles are increasing the last two decades.[1,2] *Nannizziopsis guarroi* is an ascomycetous, filamentous fungus of the family Nannizziopsiaceae (Eurotiomycetes: Onygenales) identified as a member of the *Chrysosporium* anamorph of *Nannizziopsis vriesii* complex (CANV).[3–5]

Different reports of recent years have indicated several keratinophilic CANV as a reason for often fatal superficial or deep dermatomycosis in a number of reptile species whereas human-associated species exist as well.[3,4,6,7] *Nannizziopsis guarroi* appeared mainly in central bearded dragons (*Pogona vitticeps*) and green iguanas (*Iguana iguana*) worldwide causing the so called 'Yellow fungus disease'.[3,4,6,8,9] Besides skin lesions, rare cases of systemic infection exist.[10] *Nannizziopsis chlamydospora* and *N. draconii* have recently been described in central bearded dragons with dermatomycosis.[8,11] Another primary pathogenic species of the Onygenales order is *Ophidiomyces ophiodiicola*, causing snake fungal disease.[12,13] While snake fungal disease is well known in captive snakes all over the world, it is also emerging in free-ranging snake populations across the United States.[1,14]

Cytological and histopathological examination of the skin and underlying tissue biopsy samples is advisable in order to diagnose deep fungal dermatitis.[2] Voriconazole applied orally at 10 mg/kg bodyweight once every 24 h up to 70 days is recommended as an efficient and safe treatment option of CANV-complex-induced dermatitis in bearded dragons.[15] The fungus may be difficult to identify without isolation and differentiation, because the aleurioconidia are similar to those of other *Chrysosporium* or *Trichophyton* species, and the arthroconidia resemble those of *Geotrichum* or *Trichosporon* species.[4]

Identification of these fungal species capable of inducing fatal disease is of great importance to initiate targeted antifungal therapy and reduce mortality in reptiles. Next generation sequencing is considered the gold standard for accurate identification of human pathogenic yeasts and filamentous fungi.[16] Phylogenetic studies on fungal pathogens of the CANV-complex focus on fragments of the small subunit ribosomal DNA (SSU), DNA sequencing of the nuclear ribosome regions ITS1-5.8S-ITS2, and domains 1 and 2 (D1/D2) of the large subunit ribosomal DNA (LSU), as well as actin and β-tubulin genes.[4,5,11]

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is established as a time-saving, accurate, affordable, and feasible diagnostic tool for identification of fungi and bacteria in clinical microbiology.[17–20] Species differentiation of dermatophytes grown in culture by MALDI-TOF-MS, especially *Trichophyton* species, has recently been reported as fast and very specific method.[21] Currently, identification of opportunistic pathogenic fungi can be unsatisfactory due to restrictions of commercially obtainable databases.[22] There are no reports on identification of reptile-associated fungi using MALDI-TOF MS. In order to facilitate and enhance the practicability of fungal species identification in veterinary practice, it is important to generate new and supplement existing databases.[23]

The aim of this study was to prove accuracy in identification of *N. guarroi* with MALDI-TOF MS in comparison to nucleotide sequence analysis.

Methods

Animals and sampling procedure

Nannizziopsis guarroi isolates were obtained from a total of seven lizards out of three collections; four central bearded dragons out of two collections, two green iguanas, and one European green lizard (*Lacerta viridis*) from a third collection. All lizards were adult. Central bearded dragons were bred in Germany. The other lizards came from an animal shelter; their origin is unknown. Contact between the collections was not reported. Lizards displayed a severe dermatitis, which was also seen in other members of their respective collection. Swabs from the oral cavity, tongue, trachea, skin, and cloaca were sampled sterile with individually packed sterile microbiological swabs (Applimed, Châtel-St-Denis, Freiburg, Switzerland) during the thorough clinical examination. Four of the seven lizards were euthanized due to the severity and expansion of dermatitis, and swabs were taken from the oral cavity, tongue, lungs, intestines, and liver with individually packed sterile microbiological swabs (Applimed) during post mortem examination. Skin samples as well as visceral organs from the euthanized lizards were assessed by cytological and histopathological examination followed by mycological examination of the respective swabs. The remaining three central bearded dragons, all from one collection, were treated successfully with voriconazole as recommended.[17]

Isolation, cultivation and antifungal susceptibility

The collected swabs were plated onto Sabouraud-Chloramphenicol-Gentamycin-Agar (SAB-CHL/GEN) (Oxoid, Wesel, Germany) as well as on Potato-Dextrose-Agar (PDA) (Oxoid) and incubated at 30°C. All sample cultures were stored at −70°C in cryotubes (Roti-Store cryotubes, Carl Roth, Karlsruhe, Germany) in order to preserve the isolates for further investigation. After re-cultivation of the isolates, their colonial features and growth rates were documented on SAB-CHL/GEN and PDA and incubated at 30°C and 35°C for 10 days. Growth of the cultures was observed and recorded after 5 and 10 days. Susceptibility was screened by means of disc diffusion testing. Fungal colonies were first diluted in Nutrient Broth with Glucose (Oxoid), then the fluid was distributed on SAB-CHL/GEN (Oxoid) and incubated at

35°C for 120 h. Utilized test discs included voriconazole 1 μg (CT1807B; Oxoid), fluconazole 25 μg (CT1806B; Oxoid), itraconazole 8 μg (81812 N; Neo-Sensitabs™, Rosco Diagnostica A/S, Taastrup, Denmark), nystatin 100 international units (CT0073B; Oxoid), amphotericin B 20 μg (051916048; Liofilchem®, Roseto degli Abruzzi, Italia), and terbinafine 30 μg (87412 N; Neo-Sensitabs™). Zones of inhibition surrounding the disks ≤ 30 mm were defined as resistance. In case of resistance, however, an overgrowth of the discs was mostly seen.

Molecular biological differentiation

Purification of the genomic DNA was done with a DNeasy Blood & Tissue Kit (Quiagen, Hilden, Germany) using manufacturer's protocols. Two fragments of the ribosomal gene were sequenced for genomic analysis. One of them was the SSU/ITS1-5.8S rDNA, and the second one consisted of fragments of the large subunit of the 28S rDNA (LSU) including D1/D2.[24–26] Amplification reactions were prepared using standardized polymerase chain reaction (PCR) protocols and variation of annealing temperature.[27] Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.[28] Sequences were compared with sequences listed in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).[29] The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model for SSU/ITS1-5.8S rDNA and LSU sequences.[30] Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value.

Nucleotide sequence accession numbers

The GenBank accession numbers for *N. guarroi* strain VS3603, isolated from skin lesions of a European green lizard, represent all isolates of *N. guarroi* obtained in this study, ITS1-5.8S rDNA sequence: KU342058; SSU/ITS1-5.8S rDNA sequence: KX371913; LSU gene sequence: KX371914.

MALDI-TOF MS

MALDI-TOF MS was performed using a Bruker microflex LT mass spectrometer and the MBT Compass 4.1 (Bruker Daltonik GmbH, Bremen, Germany) software. The samples were inoculated from SAB-CHL/GEN (Oxoid) or PDA (Oxoid) on Tryptone-Yeast extract-Glucose-Skim milk powder agar (TYGM) with the addition of Penicillin and Streptomycin (Institute of Bacteriology and Mycology, University of Leipzig, Leipzig, Germany). Cultures were incubated for 10 days, followed by inoculation of samples in two fluid-culture mediums, Brain-Heart-Infusion broth (BHI-B, Sifin, Berlin, Germany) and Sabouraud broth (SAB-B, Sifin), which were then incubated for one to two days at room temperature. Ten microliters of 10% Tween 80 detergent (Carl ROTH, Karlsruhe, Germany) were added to every 1 ml broth cultures and centrifuged at 6700 *g* for 10 min. The supernatant was removed; 1 ml of distilled water was added and centrifuged at 6700 *g* for 10 min again. The supernatant was again removed, and 200 μl of distilled water and 600 μl of ethanol (Carl ROTH) were added. Samples were frozen at −80°C for 12 h, after which they were defrosted then centrifuged at 6700 *g* for 5 min. The supernatant was removed, and the fungal pellets were dried under an extraction fan afterward for 30 min. The obtained pellets were resuspended in 50 μl of 1:1 70% formic acid (Carl ROTH) and 50 μl acetonitrile (Sigma-Aldrich, Munich, Germany). After centrifugation at 6700 *g* for 5 min, 1 μl of the supernatant was applied two times to a MALDI-target plate, air dried, and afterward proceeded according to the manufacturer's instructions. In each case of the isolates, sampling material was applied on 12 spots of the MALDI-target plate of each fluid medium. Calibration of the Mass Spectrometer was done using the Bruker Bacterial Test Standard (Bruker Daltonik) (BTS), a lyophilisate of *Escherichia coli*, according to the manufacturer's specification (Bacterial Test Standard, BTS, Bruker Daltonik, Bremen).

For internal validation and as a quality management criterion two additional target spots were applied in every MS-measuring run (*E. coli*, 1 μl extract - isolate from a bovine fecal sample stored in cryoconserved stocks). Positive identification of this internal control ensures a correct MALDI-TOF measuring and processing run, as well as a reliable identification of the fungal isolates.

Of each spot, a so-called sum spectrum was generated, including 240 single spectra. Each of these sum spectra is already specific for the respective fungal isolate. To create an in-house database-entry for a specific fungal isolate, the sum spectra are processed by a manufacturer's software package and result in a so-called master spectrum. Any new identification run of an unknown fungal isolate is compared to these master spectra and yields the respective score. Each fluid medium was considered separately as an own database entry.

The obtained mass spectra were compared and analyzed with flexAnalysis Version 3.4 software (Bruker Daltonik) and, based on the results, proteomic baselines and dendrograms of both fluid mediums were generated. The mass spectra (4000 to 11000 Da) were baseline corrected, straightened, equipped with integer mass numbers. The database was extended by using the MBT Compass

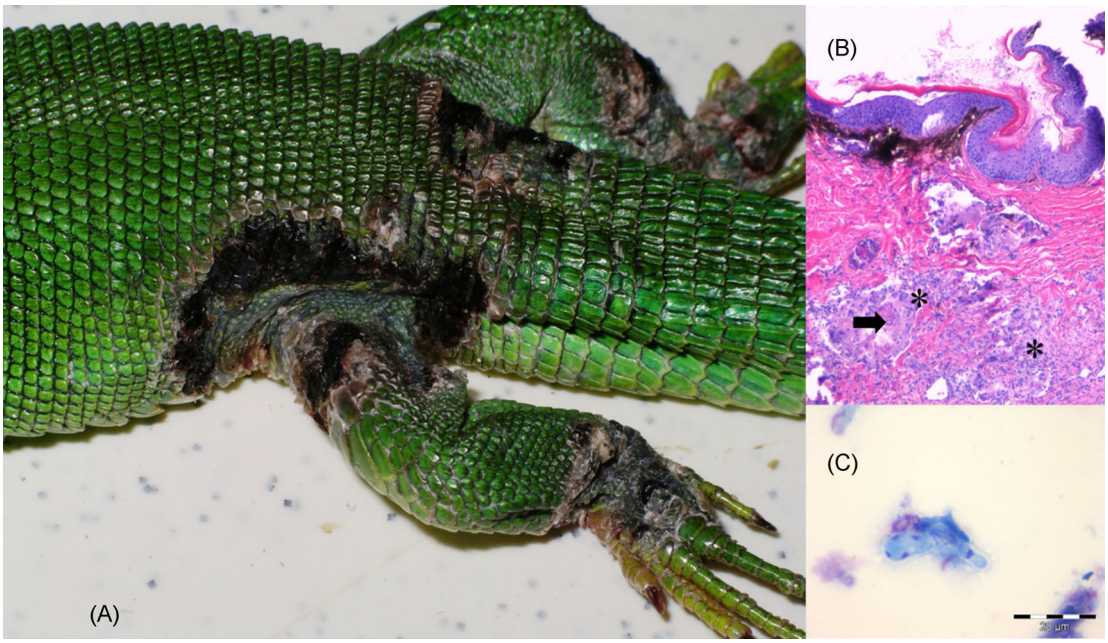

Figure 1. Yellow fungus disease in a European green lizard (*Lacerta viridis*). (A) Yellow to brown crusty extensive skin lesion of the hind legs and pelvis caused by *Nannizziopsis guarroi* infection. (B) Microphotograph presenting granulomas (asterisks) in the subcutaneous tissue consisting of central fibrin and cell detritus and fungal elements (arrow) surrounded by heterophiles, macrophages and connective tissue. H&E, 100×. (C) Pleomorphic conidia and occasionally short septate hyphae are present cytological in smears of the skin lesions. DiffQuik®, 1000×. This Figure is reproduced in color in the online version of *Medical Mycology*.

Explorer 4.1 (Bruker Daltonik). The results were presented as a ranking list of database entries, which were listed in descending mass spectra accordance. The received mass spectra were also compared with each other to investigate for identical isolates. A score value between 0 and 3.000 was calculated automatically for each mass spectra, respective target spot. According to manufacturer's recommendations, score values are classified in the categories of identifications with high-confidence identification (scores 2.00–3.00), low-confidence identification (scores 1.70–1.99) and organism identification not possible (0.00–1.69).

Results

Histopathological examination

Deep ulcerative granulomatous dermatitis presented similar in all seven lizards. Inflammatory reaction consisting of heterophiles, macrophages, and lymphocytes as well as fungal conidia (partly with budding) and short solitary undulate, occasionally septate hyphae were already detected on cytological examination of skin biopsy sample smears in all presented lizards. Histopathological examination revealed granulomas in the epidermis as well as in underlying tissues. Granulomas consisted of central fibrin and cell detritus and fungal elements surrounded by heterophiles, macrophages, and connective tissue. The epidermis often revealed ulceration with fibrin deposition, fungal hyphae, and conidia (Fig. 1). Granulomas or other findings indicative for a fungal infection were absent in visceral organs of the four necropsied lizards.

Isolation, cultivation and antifungal susceptibility

Cultivation of swabs taken from the skin lesions resulted in good growth of colonies at 30°C and 35°C. Cultures on SAB-CHL/GEN (Oxoid) measured 0.6–0.7 cm/1.6–2.0 cm in diameter, whereas on PDA (Oxoid) measured 0.8–1.1 cm/1.4–1.9 cm in diameter at 30°C after 5 days and after 10 days, respectively. Culture temperature of 35°C resulted in colonies of 0.6– 1.0/0.9–1.6 cm on SAB-CHL/GEN (Oxoid) after 5 and 10 days and in colonies of 0–1.1/0.9–1.8 cm in diameter on PDA (Oxoid). Macro- and micromorphological characteristics were comparable to the ones already described.[4] Irrespective of the isolate colonies were white, cottony, dull, flat, dry, often plicated and zonate, rarely with exudate droplets on the colony's surface and yellow to orange reverse (Fig. 2). Conidia were commonly sessile or at the ends of short hyphae. They were pleomorph with truncate bases, mostly single-celled, and occasionally two-celled (Fig. 3). *Nannizziopsis guarroi* was not isolated in swabs from the oral cavity, tongue, trachea, cloaca, and visceral organs.

While antifungal susceptibility differed between the isolates from different collections, it was identically in isolates taken from animals within one collection (Table 1).

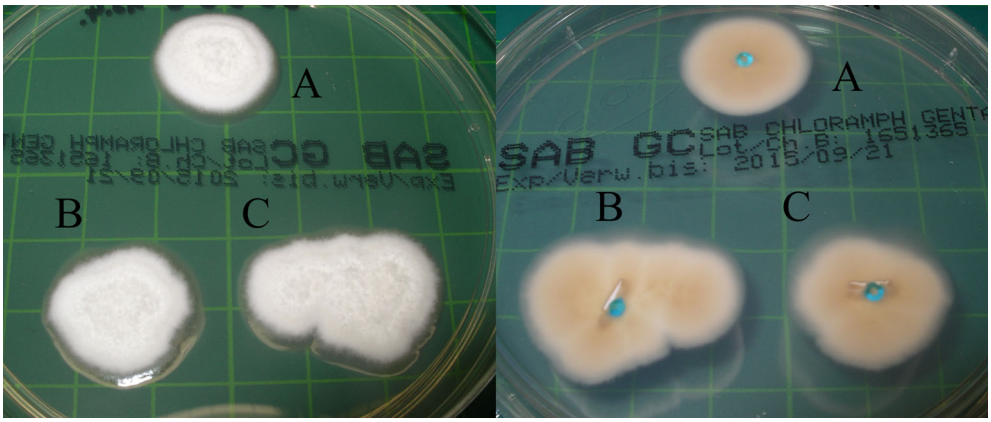

Figure 2. Colonies of three isolated *Nannizziopsis guarroi* from (A) central bearded dragon (*Pogona vitticeps*) number VRK 62124 from the group VRK 62125, VRK 62094, (B) central bearded dragon number VRK 51405, (C) European green lizard (*Lacerta viridis*) number VRK 55556 kept together with green iguanas (*Iguana iguana*) (VRK 55381, VRK 55567). Colonies, cultured on Sabouraud-Chloramphenicol-Gentamycin-Agar (SAB-CHL/GEN) (Oxoid, Wesel, Germany) after 10 days of incubation at 30°C, presented similar appearance. This Figure is reproduced in color in the online version of *Medical Mycology*.

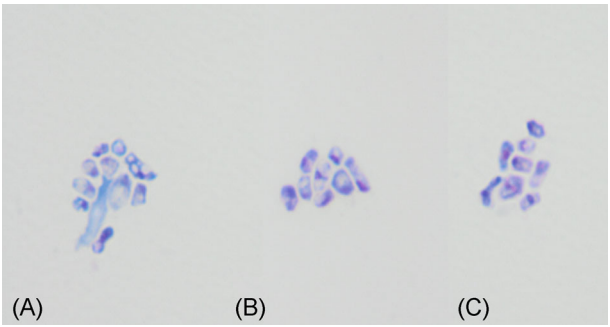

Figure 3. Microphotographs of three isolated *Nannizziopsis guarroi* from (A) central bearded dragon (*Pogona vitticeps*) number VRK 62124 from the group VRK 62125, VRK 62094, (B) central bearded dragon number VRK 51405, (C) European green lizard (*Lacerta viridis*) number VRK 55556 kept together with green iguanas (*Iguana iguana*) (VRK 55381, VRK 55567). Arthroconidia, taken from cultures on Sabouraud-Chloramphenicol-Gentamycin-Agar (SAB-CHL/GEN) (Oxoid, Wesel, Germany) after 10 days of incubation at 30°C, presented similar appearance. DiffQuik®, 1000×. This Figure is reproduced in color in the online version of *Medical Mycology*.

Molecular biological differentiation

Sequences of the seven isolates were 100% identical, so that *N. guarroi* strain VS3603, which has been stored in GenBank, represents all seven isolates. *Nannizziopsis guarroi* was proven by sequencing of the ITS1-5.8S rDNA (GenBank accession no. KU342058) and D1/D2 (GenBank accession no. KX371914) resulting in 100% identity to various described *N. guarroi* isolates (data not shown).[3,4] The fragment of the SSU revealed two single nucleotide polymorphisms to *N. guarroi* strain UAMH 10171 (GenBank accession no. KF466862.1) identical within the seven isolates.[4] Phylogenetic analysis of the SSU/ITS1-5.8S rDNA (GenBank accession no. KX371913) confirmed the isolated strain VS3603 in a clade with *N. guarroi*, and the human pathogenic *N. hominis*, *N. infrequens*, and *N. obscura* (Fig. 4).[4] Phylogenetic analysis of the LSU (GenBank accession no. KX371913) confirmed the isolated strain VS3603 in a clade with *N. guarroi*, *N. draconi* and *N. chlamydospora* (Fig. 5).[5]

Table 1. Summary of antifungal susceptibility testing of the seven *Nannizziopsis guarroi* isolates. Utilized test discs include fluconazole (FLU) 25 μg (CT1806B; Oxoid, Wesel, Germany), itraconazole (ITRAC) 8 μg (81812 N; Neo-Sensitabs™, Rosco Diagnostica A/S, Taastrup, Denmark), voriconazole (VOR) 1 μg (CT1807B; Oxoid), terbinafine (TREBI) 30 μg (87412 N; Neo-Sensitabs™), nystatin (NS) 100 international units (CT0073B; Oxoid) and amphotericin B (AMB) 20 μg (051916048; Liofilchem®, Roseto degli Abruzzi, Italia). Zones of inhibition surrounding the disks ≤ 30 mm were defined as resistance.

Reptile species/isolate number	FLU 25 μg	ITRAC 8 μg	VOR 1 μg	TERBI 30 μg	NS 100 IU	AMB 20 μg
European green lizard (*Lacerta viridis*)/55556+	S	R	R	S	S	R
Green iguana (*Iguana iguana*)/55381+, 55567+	S	R	R	S	S	R
Bearded dragon (*Pogona vitticeps*)/62094*, 62124*, 62125*	R	S	S	S	S	S
Bearded dragon/51405	S	R	S	S	R	R

*Lizards kept in one collection.
+Lizards kept in one collection.

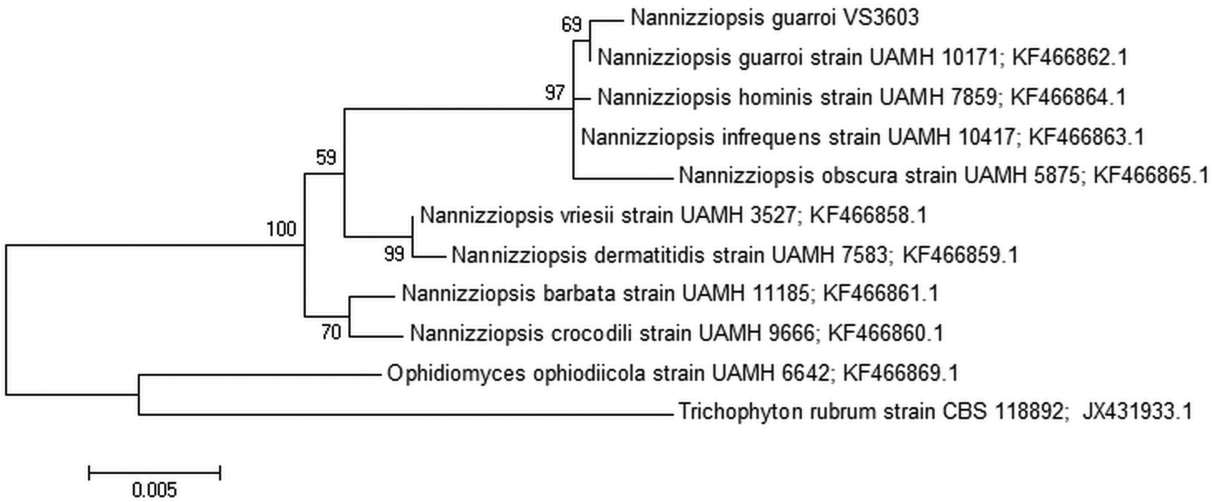

Figure 4. Maximum likelihood phylogeny inferred from the analysis of small subunit 18S rDNA (SSU)/internal transcribed spacer (ITS)1-5.8S rDNA (SSU/ITS1-5.8S) sequences of fungi of the family Nannizziopsiaceae (Eurotiomycetes: Onygenales) with *Trichophyton rubrum* and *Ophidiomyces ophiodiicola*, as member of a non-nannizziopsiaceous species in the order Onygenales, used as outgroup. The tree with the highest log likelihood (0.0000) is shown and presents four newly generated SSU/ITS1-5.8S rDNA sequences (GenBank accession number: KX371913). 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 measured in the number of substitutions per site. The analysis involved eleven nucleotide sequences. There were a total of 1598 positions in the final data set. Reference sequences were taken from the GenBank database (http://www.ncbi.nlm.nih.gov) and have been labeled with the GenBank accession numbers.

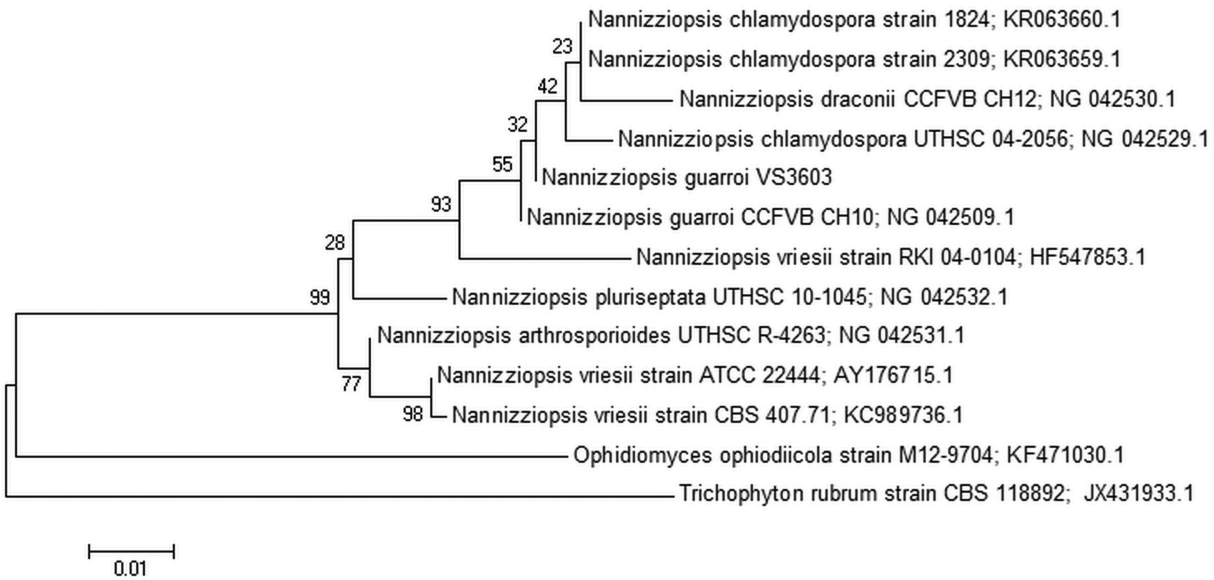

Figure 5. Maximum likelihood phylogeny inferred from the analysis of a large fragment of the large subunit of the 28S rDNA (LSU), including the domain (D)1 and D2, of fungi of the family Nannizziopsiaceae (Eurotiomycetes: Onygenales) with *Trichophyton rubrum* and *Ophidiomyces ophiodiicola*, as member of a non-nannizziopsiaceous species in the order Onygenales, used as outgroup. The tree with the highest log likelihood (0.0000) is shown and presents four newly generated LSU sequences (GenBank accession number: KX371914). 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 measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. There were a total of 552 positions in the final dataset. Reference sequences were taken from the GenBank database (http://www.ncbi.nlm.nih.gov) and have been labelled with the GenBank accession numbers.

Mass spectra analysis

The seven *N. guarroi* isolates consistently revealed characteristic peaks in their sum spectra at m/z 4747, m/z 6048, m/z 6792, m/z 9388, m/z 10429 (Fig. 6). The results of both fluid culture mediums demonstrated similar mass spectra. One isolate (14%) could be certainly related to genus and probably to species level with scores ≥ 2.0. Six of the seven isolates (86%) could be certainly identified to species level with scores ≥ 2.3 (Table 2). In consideration to results of sequence analysis, the obtained mass spectra were incorporated to our in-house database as *N. guarroi*. Dendrograms based on proteomic fingerprint analysis obtained from both culture media revealed almost comparable results within the isolates, with exception of the isolates of one green iguana. Two clusters were generated; one cluster consisted of isolates from central bearded dragons of one collection and an isolate cultured in SAB-B from a green iguana. The isolate cultured in BHI-B from this green iguana is part of the other cluster. This is also the case for isolates from the third central bearded dragon, which was kept together with the two other ones. In addition to the other green iguana and the European green lizard from the animal shelter, the single-kept central bearded dragon also clustered in the second cluster (Fig. 7).

Discussion

Morphological findings of *N. guarroi* correspond to those already reported except of the yeast-like colonies, which were absent in this study on SAB-CHL/GEN and PDA at 30°C as well as at 35°C.[4] A preference in culture media or temperature couldn't be observed. Resistance against voriconazole 1 µg test discs in all isolates from one collection is surprising as no reports of treatment failure with voriconazole exist so far.[15]

With this study, a large fragment of the LSU including D1/D2 as well as SSU/ITS1-5.8S rDNA sequences of a *N. guarroi* strain, identical in all seven isolates, are provided to database for the first time, which can be used for upcoming genomic and epidemiological analysis of fungal isolates from the Nannizziopsiaceae family. The strain was identified as *N. guarroi* according to 100% homology in ITS1-5.8S and D1/D2 to *N. guarroi* strains referenced in GenBank. Phylogenetic analysis confirmed the evolutionary phylogeny as described before.[4,5] Sequencing of actin- and β-tubulin-genes or whole genome analysis are recommended for further genetic confirmation of species identification or for epidemiological studies of those emerging pathogens.[5]

Advantages of MALDI-TOF MS as a reliable diagnostic tool for bacteria and yeasts have been widely reported in human and veterinary medicine.[19,20,23] Unknown spectra are obviously marked as unidentifiable.[31] Establishment of mycology reference databases for the MALDI-TOF MS library, as reliable diagnostic laboratory in reptile medicine, is therefore required and essential. In this study, all *N. guarroi* sequences were compared to sequences of the NCBI BLAST database (National Center for Biotechnology Information, US National Library of Medicine Basic Local Alignment Search Tool), a database with public access. Different from establishing an in-house MALDI-TOF MS database, NCBI BLAST provides many different uploaded representative sequences of fungal species. This allows a more comprehensive description of our and other *Nannizziopsis* species in a phylogenetic tree compared to a dendrogram of MS – spectra. In combination with cultural identification, histological and microscopic evaluation and the clinical signs, NCBI BLAST search shows the correct identification of a fungal species. Due to the low emergence of isolates in this mycological fringe and a very limited availability of reference strains, a comparison to our established in-house database was unfortunately the only possibility determining the score values. Any identification run of an isolate was done by excluding the respective isolate. Comparing an isolate to itself was therefore impossible. Within the scope of using reference strains of culture collections for establishing a MS database, it was unfeasible for the authors to use such strains in this study, due to reasons of limited availability. However, the authors will prospectively have a focus on that fact, as this study was also carried out for establishing a fast and reliable diagnostic method for future use. Nevertheless, accuracy and safety of MALDI-TOF MS applied as a tool for diagnosing yellow fungus disease in lizards could be acknowledged, so that this study is the first report on the use of MALDI-TOF MS for identification of the emerging reptile-associated pathogenic ascomycetous fungus *N. guarroi*. It is striking that in all of the seven isolates *N. guarroi* was identified independent of the used subculture media, with both SAB-B and BHI-B medium regularly yielding high scores. The results of both fluid culture media demonstrated similar mass spectra. There was no effect of type and composition of the culture medium on identification accuracy. Beyond that, the chemical structure of the so-called matrix-substance HCCA favours a co-crystallization with the fungal ribosomal proteins and only to a very small degree with other molecules or compounds. Looking at the mechanism of MALDI-TOF mass spectrometry, a co-crystallized mixture of HCCA-matrix and (ribosomal) fungal proteins is spotted on the target, irradiated by UV-laser light and accelerated by high voltage as ionized protein-fragments in a vacuum flight tube. Resulting from the mass differences between the protein-fragments and thereby different times of flight, a specific fungal peak pattern is detectable. To

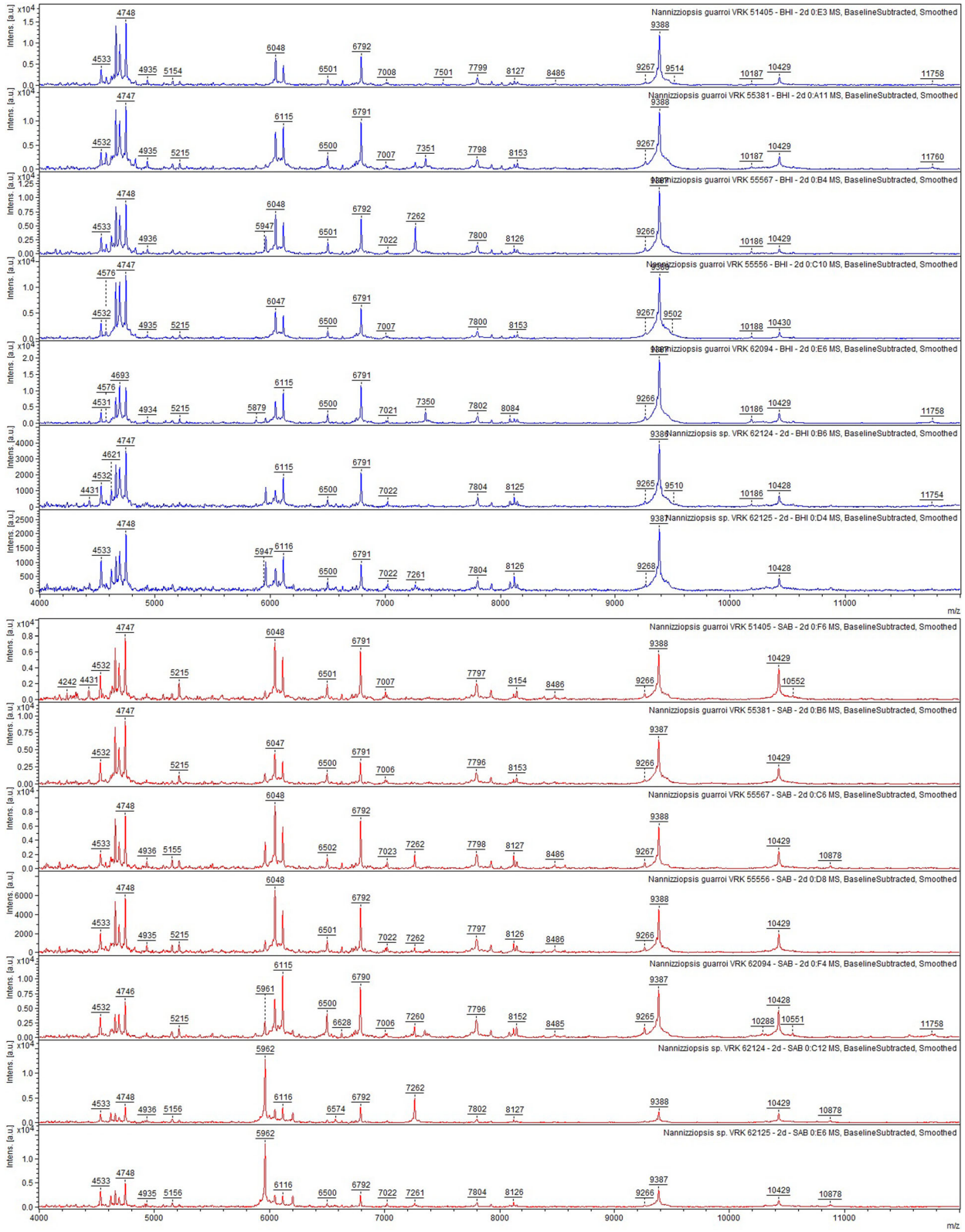

Figure 6. Mass spectra of seven *Nannizziopsis guarroi* isolates from skin lesions of central bearded dragons (*Pogona vitticeps*) (VRK 51405, VRK 62094, VRK 62124, VRK 62125), green iguanas (*Iguana iguana*) (VRK 55381, VRK 55567), and a European green lizard (*Lacerta viridis*) (VRK 55556) after subcultivation in Brain-Heart-Infusion broth (BHI-B) and Sabouraud broth (SAB-B) generated by use of a Bruker microflex LT mass spectrometer and the MBT Compass 4.1 (Bruker Daltonik GmbH, Bremen, Germany) software. The absolute ion intensities are shown on the *Y* axis and the masses (m/z) on the *X* axis. This Figure is reproduced in color in the online version of *Medical Mycology*.

Table 2. Proteomic analysis results of *Nannizziopsis guarroi* identified by based on Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) presented mainly with high-confidence identification (scores 2.00–3.00), except for two spots with low-confidence identification (scores 1.70–1.99).

Reptile species	Isolate number		Score range (number of analytes) after incubation at Sabouraud broth (max. 12 spots possible)	Score range (number of analytes) after incubation at Brain-Heart-Infusion broth (max. 12 spots possible)
European green lizard	VRK 55556+	#	1.951– 2.490 (10)	2.036–2.389 (9)
(*Lacerta viridis*)		#[1]	1.951	
		##	1.621–2.042 (10)	1.652–2.025 (9)
	VRK 55381+	#	2.153–2.385 (12)	2.112–2.397 (10)
Green iguana		##	1.553–1.933 (12)	1.814–2.163 (10)
(*Iguana iguana*)	VRK 55567+	#	2.184–2.428 (12)	2.113–2.340 (10)
		##	1.792–2.020 (12)	1.753–2.198 (10)
	VRK 62094*	#	2.052–2.295 (12)	2.129–2.290 (12)
		##	1.644–1.972 (12)	1.570–2.112 (12)
	VRK 62124*	#	1.927–2.447 (9)	2.142–2.444 (9)
Bearded dragon		#[1]	1.927	
(*Pogona vitticeps*)		##	1.840–2.424 (9)	2.104–2.432 (9)
	VRK 62125*	#	2.239–2.447 (4)	2.103–2.405 (5)
		##	2.139–2.399 (4)	2.091–2.334 (5)
	VRK 51405	#	2.187–2.359 (10)	2.187–2.431 (12)
		##	1.685–1.972 (10)	1.6200–2.003 (12)

*Lizards kept in one collection.
+Lizards kept in one collection.
#Log(score) value range best matches (number of detected spots on the MALDI-TOF MS-target plate).
#[1]Spots with low-confidence identification (scores 1.70–1.99) in the sector of all best matches. These spots haven't been consulted for the generation of master spectra but were considered as outliers and have been sorted out.
##Log(score) value range second best matches (number of detected spots on the MALDI-TOF MS-target plate).

the best of our knowledge, with this experimental design we can exclude significant interference patterns between residues of the liquid medium and the fungal proteins. In our understanding, clustering effects are caused by different growing conditions, like changes in liquid media and are in a further sense a possible important outcome of this study. Contrary to genomic analysis, proteomic analysis reveals two clusters of isolates irrespective of the origin of the isolate or the antifungal susceptibility. Influence of liquid media was shown in one isolate, which was presented in both clusters. However, epidemiological studies based on proteomic analysis seem to be unsuitable, as isolates varies inside one collection.

Six of the seven diseased lizards were housed together in two different collections and suffered from outbreaks of yellow fungus disease as part of a flock disease, so that crowding, stress, or suboptimal temperature can be discussed as a possible cause of immunosuppression in the diseased lizards described here. This is in accordance to other reports on yellow fungus disease outbreaks.[11,32] Koch's postulates have been fulfilled experimentally in veiled chameleons; hence, *Nannizziopsis guarroi* can be expected to act as an obligate pathogenic saprophytic fungus in other lizards as well.[33] The pathogenicity is comparable with snake fungal disease. Superficial damage to the outer epidermal layers may serve as the primary route of infection, as it has experimentally been demonstrated.[12,13,33]

Yellow fungus disease in European green lizard has not been reported previously. An impact on the native lizard population in Europe, comparable to snake fungal disease in native American snakes, seems possible.[14] Infection of the European green lizard as described here was most likely caused by transmission of *N. guarroi*-contaminated soil from infected green iguanas, which were housed in the same room and presented with yellow fungus disease at the same time. Snake fungal disease and/or yellow fungal disease in free-ranging lizard or snake populations from Europe have not been detected so far.

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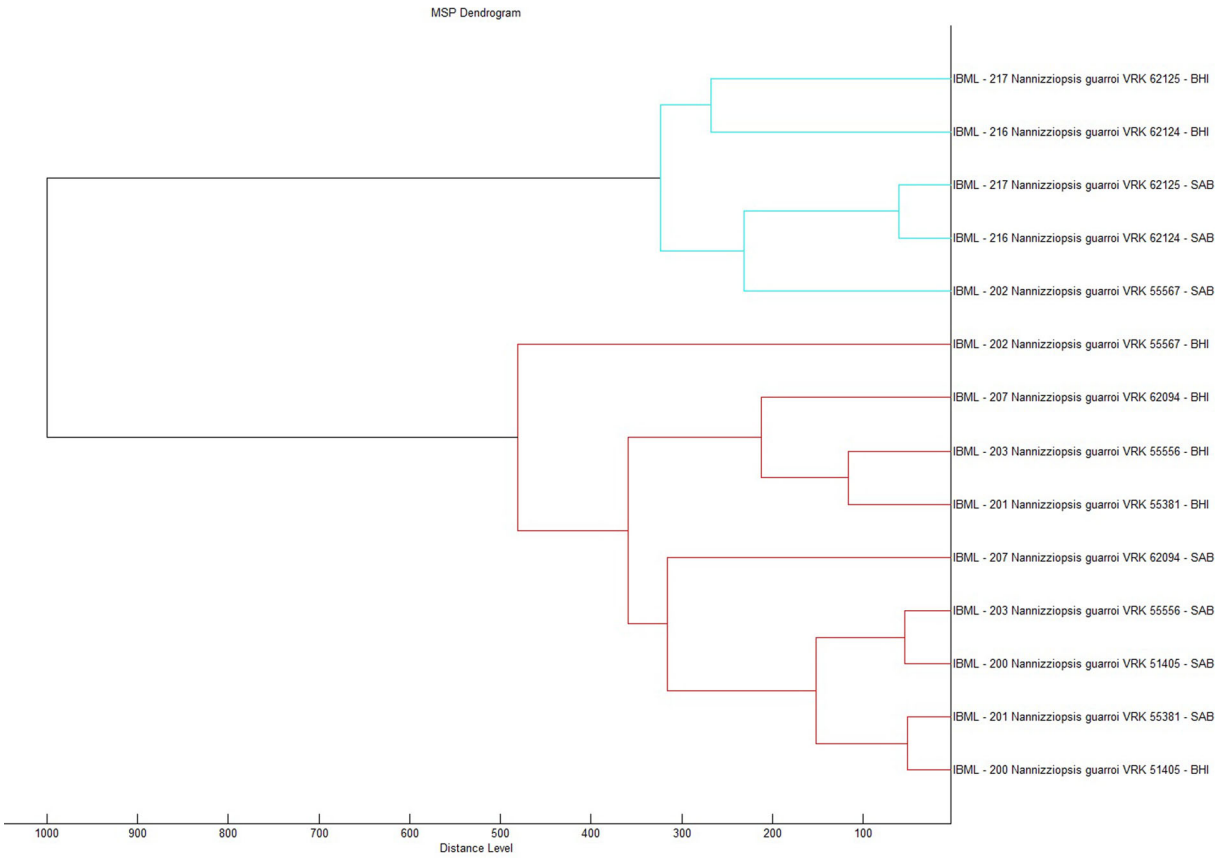

Figure 7. Principal component analysis (PCA) dendrogram generated by MALDI Biotyper mass spectra of 14 *Nannizziopsis guarroi* isolates in Brain-Heart-Infusion broth (BHI-B) and Sabouraud broth (SAB-B) from central bearded dragons (*Pogona vitticeps*) (VRK 51405, VRK 62094, VRK 62124, VRK 62125), green iguanas (*Iguana iguana*) (VRK 55381, VRK 55567) and a European green lizard (*Lacerta viridis*) (VRK 55556) with yellow fungus disease. This Figure is reproduced in color in the online version of *Medical Mycology.*

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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